

# **Induced Human Pluripotent Stem Cell – derived NK Cells as an Alternative Source of Lymphocytes for Anti-Cancer Immunotherapy**

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SUSANNE O'BRIEN

(nee Arthold)

A thesis submitted for the degree of

Doctor of Philosophy

UCL

## **DECLARATION**

I, Susanne O'Brien, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## ABSTRACT

Human induced pluripotent stem cells (hiPSCs) hold great promise in regenerative medicine as they have the potential to differentiate into any specialized cell type and retain unlimited self-renewal capacity *in vitro*. The use of hiPSC-derived lymphocytes for adoptive cell transfer therapies is appealing as it provides a potentially indefinite cell source and a platform for genetically modifying cells. Natural killer (NK) cell-based therapy has shown promising results in the treatment of haematological malignancies and the unlimited derivation of NK cells from hiPSCs could overcome the current obstacle of insufficient NK cell numbers for cellular anti-cancer immunotherapy. In this study, we initially used a two-stage culture system to differentiate human pluripotent stem cells (hPSCs) into NK cells. First, haematopoietic stem cells (HSCs) were derived by co-culture with murine OP9 stromal cells. The CD34-expressing fraction was enriched with magnetic activated cell sorting (MACS) and phenotypically characterised by cell surface marker expression. Colony forming unit (CFU) assay confirmed that hPSC-derived CD34<sup>+</sup> cells had multi-lineage differentiation potential. Subsequently, HSCs were co-cultured with murine EL08.1D2 feeder cells and under feeder-free conditions with GBGM medium to generate NK like cells. Interestingly, we observed a 100-fold lower cell expansion of hiPSC-derived CD34<sup>+</sup> cells in NK cell differentiation conditions compared to umbilical cord blood (UCB)-derived CD34<sup>+</sup> cells. Despite this observation the phenotype of hiPSC-derived NK like cells was comparable to UCB CD34<sup>+</sup>-derived NK cells and freshly isolated peripheral blood natural killer cells (PBNKs). As an alternative to the two-stage culture system, we aimed to drive NK cell differentiation by overexpressing the NK cell-specific transcription factor E4bp4 during *in vitro* derivation. Our data suggest that the overexpression of E4bp4 does not improve the efficiency of hiPSC-derived NK like cell differentiation, nor can E4bp4 alone induce the NK cell-specific transcriptional network in fibroblasts.

## ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisor Professor Amit Nathwani for his help and support during my PhD project. Thank you for guiding me through the ups and downs, with your support and best intentions. I would also like to thank my secondary supervisor Professor Mark Lowdell and Dr. Aurora Saudemont for constructive discussions, advice on my project and for all their help and support.

I am very grateful that I got the Marie Skłodowska – Curie ESR fellowship awarded and could partake in the “NaturIMMUN” ITN programme – working on NK cell-based anti-cancer immunotherapies. A special thank you to the organisers Professor Erhard Hofer and Professor Ulrike Koehl, whose determination and hard work made this programme a very special experience for me. Thanks to all the participants for fruitful discussions and feedback on my work and results. Also a very big thank you to all the fellow students and post docs in the programme who made the meetings extremely enjoyable. Altogether, this programme offered great networking opportunities and broadened my career-horizon tremendously.

A big thank you to everyone in my research group and in the department for all the help and advice. A special thank you to former and current lab members of the hiPSC research group, including Pollyanna Goh, Sajjida Jaffer, Sara Caxaria, Mahnaz Abbasian and Ariane Chapgier for all the scientific and especially emotional support! A special thank you to Sajjida Jaffer for all the support during the project and for guiding me through to the end of my PhD. Furthermore, I would like to thank Pollyanna Goh and Dominic Sweeney for making time to give me final comments on my thesis. I would like to thank my husband James and my stepdaughter Esme for non-scientific support and for cheering me up whenever necessary. Last but not least I would like to thank all my friends and family members for giving me all the support needed for completing this project.



## TABLE OF CONTENTS

DECLARATION .....	2
ABSTRACT .....	3
ACKNOWLEDGEMENT .....	4
LIST OF FIGURES .....	11
ABBREVIATIONS.....	14
Chapter 1 Introduction .....	18
1.1 Tissue-specific NK cells.....	19
1.2 Target recognition by NK cells .....	22
1.3 NK cell receptor repertoire .....	25
1.3.1 Activating and Inhibitory receptors.....	25
1.3.2 DNAM-1 receptor.....	29
1.3.3 TIGIT .....	30
1.3.4 Cytotoxic and regulatory T cell molecule (CRTAM).....	30
1.4 NK cell effector functions .....	31
1.4.1 Secretion of cytokines.....	33
1.4.2 Release of perforin and granzyme .....	33
1.4.3 Killing through apoptotic pathways using FasL and TRAIL .....	34
1.5 Human NK Cell Development .....	35
1.5.1 Transcriptional control of ILC development.....	38
1.6 NK cells and Immunotherapy.....	40
1.6.1 Challenges of NK cell immunotherapy .....	43
1.6.2 NK cell engineering .....	43
1.6.3 NK cell sources for immunotherapy .....	44
1.7 Stem cells.....	46
1.7.1 Embryonic stem cells.....	47
1.7.2 Induced pluripotent stem cells .....	48
1.7.3 Reprogramming methods .....	49

1.7.4	Reprogramming cocktail .....	51
1.7.5	Clinical application .....	51
1.7.6	Challenges of hiPSC based therapy .....	54
1.8	Research proposal.....	56
1.8.1	Aim 1 - To generate and characterize haematopoietic progenitors from hESCs and hiPSCs.....	56
1.8.2	Aim 2 - To establish a robust <i>in vitro</i> differentiation protocol for NK cell derivation from hPSC-derived haematopoietic progenitors.....	57
1.8.3	Aim 3 - To explore transcription factor – driven NK cell differentiation by overexpression of E4bp4.....	58
Chapter 2	Materials & Methods.....	59
2.1	Cell culture.....	59
2.1.1	Human pluripotent stem cells.....	60
2.1.2	OP9 feeder cells .....	61
2.1.3	EL08.1D2 feeder cells.....	62
2.1.4	Isolation of human mononuclear cells from peripheral blood and umbilical cord blood .....	63
2.1.5	Magnetic activated cell sorting (MACS).....	63
2.1.6	Co-culture with OP9 feeder cells.....	64
2.1.7	Serum- and feeder free differentiation with cytokines.....	65
2.1.8	Embryoid body formation .....	66
2.1.9	Spin embryoid body formation .....	66
2.1.10	NK cell differentiation .....	67
2.2	Phenotypic analysis by flow cytometry .....	68
2.3	Colony forming unit assay .....	70
2.4	CD107a degranulation assay.....	71
2.5	Molecular Biology .....	71
2.5.1	RNA isolation.....	71
2.5.2	cDNA Synthesis.....	72

2.5.3	qRT-PCR.....	73
2.5.4	Protein extraction.....	76
2.5.5	BCA assay.....	76
2.5.6	Western Blotting .....	78
2.5.7	Cloning .....	79
2.5.8	Restriction digest .....	80
2.5.9	Quick ligation.....	81
2.5.10	PCR protocol for cloning.....	82
2.5.11	Sequencing .....	82
2.6	Lentivirus work.....	83
2.6.1	Transformation .....	83
2.6.2	Transient transfection .....	83
2.6.3	Preparation of Lentiviral particles.....	83
2.6.4	Determination of lentiviral titer .....	84
2.6.5	hiPSC transduction.....	85
2.6.6	Statistical analysis .....	85
Chapter 3 Generation and characterisation of haematopoietic progenitors from hPSCs 86		
3.1	Introduction.....	86
3.1.1	Haematopoietic stem cell specification <i>in vivo</i> .....	86
3.1.2	<i>In vitro</i> HSC differentiation from pluripotent stem cells.....	87
3.1.3	Challenges and obstacles of hiPSC-derived HSC derivation .....	89
3.2	Results .....	91
3.2.1	Haematopoietic stem cell progenitors derived with co-culture on murine OP9 stromal cells.....	91
3.2.2	CD34 <sup>+</sup> cells can be generated from hPSCs .....	93
3.2.3	Feeder- and serum-free HSC differentiation with APEL medium .....	94
3.2.4	Embryoid body formation.....	98

3.2.5	CD34 <sup>+</sup> cell enrichment and immunophenotype characterisation.....	98
3.2.6	HiPSC-derived CD34-expressing cells compared to UCB-derived CD34 <sup>+</sup> cells .....	103
3.2.7	Assessment of myeloid differentiation potential with colony forming unit (CFU) assays .....	105
3.2.8	Transcriptional control during HSC differentiation in OP9 co-culture	108
3.3	Discussion .....	115
Chapter 4 Generation of hiPSC- derived NK cells and their characterisation .....		120
4.1	Introduction .....	120
4.2	Results.....	124
4.2.1	NK cell derivation from hPSC-derived CD34 <sup>+</sup> cells with the mouse fetal liver cell line EL08.1D2 co-culture system .....	124
4.2.2	HPSC-derived CD34 <sup>+</sup> differentiate into CD56 <sup>+</sup> CD3 <sup>-</sup> NK like cells ....	125
4.2.3	Immunophenotypic characterisation of UCB- and hPSC-derived CD34 <sup>+</sup> cells as starting cell population for NK cell differentiation .....	133
4.2.4	CD56 is upregulated during differentiation .....	134
4.2.5	Cell numbers and expansion during NK cell differentiation .....	137
4.2.6	NK cell specific markers are expressed in hPSC-derived cells.....	138
4.2.7	MMC hiPSC-derived NK-like cells exhibit cytotoxic function against the leukemic cell line K562 in CD107a degranulation assay .....	140
4.2.8	Serum- and feeder free NK cell differentiation with GBGM medium	141
4.2.9	Immunophenotypic characterisation of UCB- and hPSC-derived CD34 <sup>+</sup> cells as starting cell population for NK cell differentiation with GBGM medium	142
4.2.10	Cell numbers and expansion during NK cell differentiation .....	144
4.2.11	HiPSC-derived NK like cells display surface marker expression close to UCB-derived NK cells and freshly isolated PBNKs.....	149
4.3	Discussion .....	155
Chapter 5 Transcription factor-driven NK cell differentiation with E4bp4 .....		159
5.1	Introduction .....	159

5.2	Results .....	162
5.2.1	Virus titration .....	163
5.2.2	UCB-derived CD34 <sup>+</sup> cells can be efficiently transduced.....	165
5.2.3	Lentiviral transduced UCB-derived NK cells keep tdTomato expression upregulated during NK cell differentiation and surface marker expression differs compared to untransduced UCB-derived NK cells .....	167
5.2.4	Cloning of new lentiviral vector into pCCL.EF1α.eGFP backbone...	171
5.2.5	E4bp4 over-expression with pCCL.EF1α.E4bp4.IRES.eGFP can be detected on RNA and protein level .....	172
5.2.6	Direct differentiation of hiPSCs to NK cells by over-expression of E4bp4	174
5.2.7	Can the over-expression of E4bp4 in hiPSCs improve the efficiency of NK cell differentiation? .....	174
5.2.8	Good transduction efficiency of hiPSCs and GFP upregulation is maintained during cell expansion in pluripotent culture conditions .....	175
5.2.9	CD34 <sup>+</sup> cell derivation from transduced hiPSCs with OP9 co-culture	176
5.2.10	E4bp4 overexpression does not improve cell expansion in NK cell differentiation .....	179
5.2.11	E4bp4 is consistently upregulated during NK cell differentiation .....	180
5.2.12	E4bp4 transduced hiPSC-derived NK like cells differ in surface marker expression compared to untransduced and mock transduced cells .....	181
5.2.13	Can fibroblasts be driven towards the NK cell lineage by over-expression of E4bp4 and culture in GBGM NK cell differentiation conditions?	185
5.2.14	E4bp4 stays upregulated during cell culture .....	187
5.2.15	CD56 and IL-15RB are not upregulated in E4bp4 transduced cells	188
5.3	Discussion .....	192
Chapter 6 General Discussion and Outlook .....		196
REFERENCES .....		202

## LIST OF TABLES

Table 1.1 NK cell inhibitory receptors.....	26
Table 1.2 NK cell activating receptors.....	28
Table 1.3 Overview of recent NK cell clinical trials with in vitro activated NK cells..	42
Table 1.4 Clinical trials with hPSCs.....	54
Table 2.1 Cell lines.....	60
Table 2.2 Antibodies used for Flow cytometry.....	70
Table 2.3 Reagents used in Molecular Biology.....	71
Table 2.4 cDNA Synthesis Mix.....	73
Table 2.5 List of primers.....	75
Table 2.6 List of standards used in BCA assay.....	77
Table 2.7 List of restriction enzymes.....	81
Table 2.8 List of sequencing primers.....	82
Table 2.9 Mix 1 of third generation lentivirus production.....	84

## LIST OF FIGURES

Figure 1.1 Flow cytometric analysis of CD56bright and CD56dim natural killer (NK) cells. ....	19
Figure 1.2 Target cell recognition by NK cells. ....	24
Figure 1.3 Effector functions of innate lymphoid cells (ILCs). ....	32
Figure 1.4 Schematic overview of human NK cell development. ....	36
Figure 1.5 Schematic overview of transcriptional control in ILC development. ....	39
Figure 1.6 The developmental potential and epigenetic states of cells at different stages of development. ....	46
Figure 1.7 Application of hiPSC technology. ....	52
Figure 3.1 Experimental layout of OP9 co-culture differentiation protocol ....	92
Figure 3.2 Morphological changes in OP9 co-culture at days 0, 6 and 9. ....	92
Figure 3.3 CD34 efficiencies with OP9 co-culture. ....	93
Figure 3.4 CD34 efficiencies with serum- and feeder free differentiation. ....	95
Figure 3.5 Pluripotency and haematopoietic marker expression. ....	97
Figure 3.6 Pan human and pluripotency marker expression. ....	101
Figure 3.7 CD43 and CD45 co-expression. ....	102
Figure 3.8 Haematopoietic cell surface marker expression. ....	105
Figure 3.9 Myeloid and erythroid progenitors form in CFU assays. ....	108
Figure 3.10 Gene expression of the 3 germ layers. ....	109
Figure 3.11 Expression of haematopoietic genes. ....	111
Figure 3.12 Expression of mesenchymal and endothelial genes. ....	112
Figure 3.13 A model of lineage differentiation from the pluripotent stem cell state. ....	113
Figure 4.1 Schematic overview of NK cell derivation methods from hPSCs. ....	122
Figure 4.2 Schematic overview of the EL08.1D2 co-culture protocol for NK cell differentiation <i>in vitro</i> . ....	124
Figure 4.3 hPSC-derived CD34 <sup>+</sup> cells for NK cell differentiation on EL08.1D2 co-culture. ....	126

Figure 4.4 Day 29 NK cell differentiation with hPSC-derived CD34 <sup>+</sup> cells (OP9 co-culture derived) .....	129
Figure 4.5 Day 49 NK cell differentiation with JOM hiPSC-derived CD34 <sup>+</sup> cells (OP9 co-culture derived). .....	130
Figure 4.6 Day 29 NK cell differentiation with hPSC-derived CD34 <sup>+</sup> cells (APEL derived).....	132
Figure 4.7 CD34 cell purities for EL08.1D2 NK cell differentiation.....	134
Figure 4.8 CD56 upregulation during EL08.1D2 NK cell differentiation. ....	136
Figure 4.9 Total fold expansion of cells during EL08.1D2 NK cell differentiation. .	137
Figure 4.10 UCB- and hiPSC-derived NK-like cells on day 34 of NK cell differentiation compared to freshly isolated PBNKs.....	139
Figure 4.11 CD107a degranulation assay MMC-derived NK like cells day 35. ....	140
Figure 4.12 Schematic overview of serum- and feeder free NK cell differentiation with GBGM medium.....	142
Figure 4.13 CD34 cell purities for GBGM NK cell differentiation.....	143
Figure 4.14 Total fold expansion of cells during EL08.1D2 NK cell differentiation.	144
Figure 4.15 In vitro generated NK cells from hPSC-derived CD34 <sup>+</sup> cells on day 28 and 35 of feeder- and serum free GBGM differentiation method. ....	147
Figure 4.16 <i>In vitro</i> generated NK cells from UCB-derived CD34 <sup>+</sup> cells on day 28 and 35 of feeder- and serum free GBGM differentiation method. ....	149
Figure 4.17 <i>In vitro</i> generated NK like cells on day 42 of feeder- and serum free GBGM differentiation method.....	154
Figure 5.1 Fibroblast-derived members of the iClub. (Pfaff and Cantz 2013) .....	161
Figure 5.2 Schematic plasmid map of original lentivirus over-expression vectors.	162
Figure 5.3 Restriction digest with BamHI.....	163
Figure 5.4 Virus titration by HEK 293T transduction efficiency. ....	164
Figure 5.5 Transduction efficiency of UCB-derived CD34 <sup>+</sup> cells. ....	166
Figure 5.6 <i>In vitro</i> generated NK cells from UCB-derived CD34 <sup>+</sup> cells on day 27 of feeder- and serum free GBGM differentiation method.....	170
Figure 5.7 Plasmid maps of newly cloned lentivirus over-expression vectors. ....	171



Figure 5.8 Restriction digest of E4bp4 overexpression plasmid DNA with SmaI and BstBI.....	172
Figure 5.9 Lentivirus construct successfully upregulates E4bp4 in Jurkat cells, HEK293T and hiPSCs. ....	173
Figure 5.10 Schematic overview of experimental layout – E4bp4 overexpression in 2-step NK cell differentiation from hiPSCs. ....	175
Figure 5.11 MMC hiPSC transduction efficiency. ....	176
Figure 5.12 CD34 expression efficiency on day 9 of OP9 co-culture differentiation. ....	177
Figure 5.13 CD34 MACS enrichment efficiency of OP9 co-culture derived CD34 <sup>+</sup> cells. ....	178
Figure 5.14 Total cell numbers during NK cell differentiation. ....	179
Figure 5.15 Relative expression of E4bp4 during <i>in vitro</i> NK cell differentiation. ..	180
Figure 5.16 <i>In vitro</i> generated NK cells from MMC hiPSC-derived CD34 <sup>+</sup> cells on day 51 of feeder- and serum free GBGM differentiation method. ....	185
Figure 5.17 Schematic overview of transdifferentiation. ....	186
Figure 5.18 Transduction efficiency day 3 post-transduction of fibroblasts.....	186
Figure 5.19 Relative expression of E4bp4, Tox and Id2 in fibroblasts over-expressing E4bp4 and cultured in NK cell differentiation medium. ....	188
Figure 5.20 Relative expression of CD56 and IL-15 RB in fibroblasts over-expressing E4bp4 and cultured in NK cell differentiation medium. ....	190
Figure 5.21 FACS analysis of GFP- and CD56-expressing cells on day 14 in adherent cells. ....	191

## ABBREVIATIONS

aAPC	Artificial antigen presenting cell
ADCC	Antibody dependent cellular cytotoxicity
AMD	Age-related macular degeneration
AML	Acute myeloid leukemia
APC	Allophycocyanin
APHP	Assistance Publique –Hôpitaux de Paris
BAT-3	HLA-B-associated transcript 3
BFU-E	Burst-forming–unit erythrocyte
BM	Bone marrow
CAR	Chimeric antigen receptor
CBMCs	Cord blood mononuclear cells
CFU-E	Colony-forming-unit erythrocyte
CFU-G	Colony-forming-unit granulocyte
CFU-GEMM	Colony-forming-unit granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony-forming-unit granulocyte, macrophage
CFU-M	Colony-forming-unit macrophage
CHILP	Common progenitor to all helper-like innate lymphoid cells
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus infection
DLI	Donor lymphocyte infusion
EB	Embryoid body

EDTA	Ethylenediaminetetraacetic acid
Eomes	Eomesodermin
EPO	Erythropoietin
FACS	Fluorescence activated cell sorting
FcyR	Fcy receptor
FITC	Fluorescein isothiocyanate
Flt3-L	FMS-like tyrosine kinase 3 ligand
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HE	Hemogenic endothelium
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell
HLA	Human leukocyte antigen
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
HSCT	Haploidentical stem cell transplantation
ICM	Inner cell mass
Id2	Inhibitor of DNA binding 2
IL-15	Interleukin-15
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-6	Interleukin-6

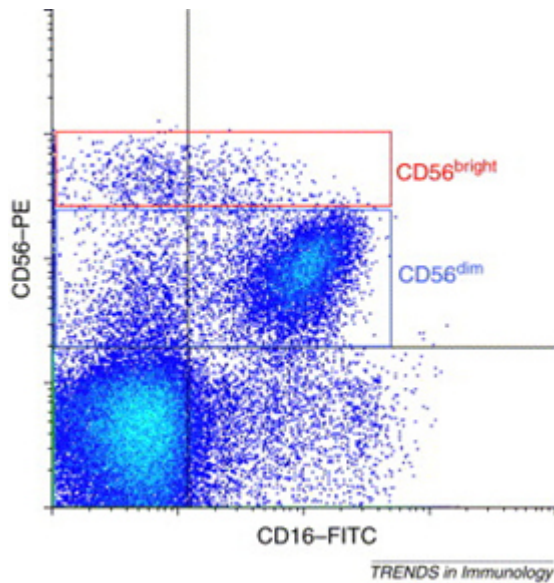
IL-7	Interleukin-7
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Intracellular immunoreceptor tyrosine-based inhibitory motifs
IU	Infectious units
IVF	<i>In vitro</i> fertilisation
KIR	Killer immunoglobulin-like receptor
MACS	Magnetic activated cell sorting
M-CSF	Macrophage colony-stimulating factor
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MM	Multiple myeloma
MOI	Multiplicity of infection
NCR	Natural cytotoxicity receptor
Nfil3	Nuclear factor interleukin 3 regulated protein
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	R-phycoerythrin
PS	Primitive streak
PVR	Polio virus receptor
RPE	Retinal pigment epithelial
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction

SCF	Stem cell factor
Tbx21	T-box 21
TCR	T cell receptor
TF	Transcription factor
TPO	Thrombopoietin
TRAIL	TNF-related apoptosis-inducing ligand
UCB	Umbilical cord blood

## Chapter 1 Introduction

Immunotherapy aims to activate the body's own immune system to fight cancer cells and to obtain long-lasting response in cancer patients. In addition to conventional therapies such as surgery, radio- and chemo-therapy, new approaches to hinder the reoccurrence of cancer cells need to be addressed. In recent years enormous advances have been made in fighting cancer by the exploitation of different components of the immune system.

NK (natural killer) cells have originally been described as CD56 positive, CD3 negative lymphocytes of the innate immune system, which recognise and kill virus-infected and tumour cells without prior sensitisation to antigens (Kiessling, Klein et al. 1975). NK cells comprise 5-15% of mononuclear cells in peripheral blood. Circulating NK cells have been divided into two main subsets: CD56<sup>bright</sup> and CD56<sup>dim</sup> based on the intensity of CD56 expression (Lanier, Phillips et al. 1986) (Figure 1.1). Each subset has distinct phenotypic properties. The majority (~90%) of circulating NK cells have low CD56 expression (CD56<sup>dim</sup>) and express CD16 (Fcγ Receptor III) whereas the CD56<sup>bright</sup> CD16<sup>-</sup> population mainly secretes cytokines to support the adaptive immunity. However, recent findings suggest that the functional and phenotypic diversity of mature NK cells is much broader than expected so far.



**Figure 1.1 Flow cytometric analysis of CD56<sup>bright</sup> and CD56<sup>dim</sup> natural killer (NK) cells.** CD56<sup>bright</sup> NK cells comprise of between 5-15% of human peripheral blood NK cells and are CD16<sup>-</sup> or CD16<sup>dim</sup>. The majority (around 90%) of NK cells is CD56<sup>dim</sup> and expresses CD16. (Cooper, Fehniger et al. 2001).

### 1.1 Tissue-specific NK cells

A significantly large number of NK cells is found in the spleen, liver, gut, and uterus (Freud and Caligiuri 2006) (Shi, Ljunggren et al. 2011). Recent studies of NK cell populations from various organs have identified distinct, tissue-specific populations of NK cells.

The gastrointestinal tract harbours a unique range of lymphocytes responsible for specific immunological requirements in the intestine. Intestinal lymphocytes include conventional single-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as unique populations of double positive and double-negative T cells (Tait Wojno and Artis 2012).

Intestinal NK cells, from mice and humans, include a distinct subset of cells known as NK22 cells that is identified by the expression of NKp46 which is known to have a role in tissue remodelling (Cella, Fuchs et al. 2009). Neither human nor murine mucosal NK22 cells produce IFN- $\gamma$  or perforins and exhibit reduced cytotoxicity (Colonna 2009). Additionally, they express the mucosa-homing chemokine receptor CCR6 and TF Ror $\gamma$ t consistent with their localisation (Cella, Fuchs et al. 2009) (Williams 2006).

Pulmonary NK cells play a major role in pathology of several respiratory conditions, including influenza, asthma, tuberculosis, and others. The maintenance of NK cells in the mucosal layers of lungs is dependent on IL-15, produced by the alveolar macrophages, as shown in murine models, or by human bronchial epithelial cells, as shown *in vitro* (Allavena, Giardina et al. 1997) (Ge, Nishioka et al. 2004). Several reports have shown that pulmonary NK cells are recruited from the periphery to the lungs upon infection; however, evidence of tissue-specific pulmonary NK cells, phenotypically distinct from those found in the blood, is also accumulating (Wang, Li et al. 2012). In murine, 70% of lung NK cells express low levels of CD27 and high levels of CD11b and are capable of producing high levels of IFN- $\gamma$ . They also express higher levels of inhibitory CD94/NKG2A and Ly49 receptors, which facilitate steady-state tolerance of the lung NK cells to daily exposure to non-pathogenic agents (Wang, Li et al. 2012). In humans, less is known about pulmonary NK cells, although NK cells isolated from pleural effusion of *Mycobacterium tuberculosis*-infected patients were CD56<sup>bright</sup>CD16<sup>low</sup>perforin<sup>low</sup> and capable of producing high levels of IFN- $\gamma$  (Schierloh, Yokobori et al. 2005).

The liver also hosts significant populations of NKR-expressing lymphoid cells including CD8<sup>+</sup> T as well as classical NK cells, reflecting its requirement to fight hepatotropic infections, as well as malignancy and metastases. Hepatic NK cells



also known as “pit” cells comprise 30-50% of the lymphocyte compartment (Norris, Collins et al. 1998). Human liver-specific NK cells are mainly CD56<sup>bright</sup>CD16<sup>-</sup> and express high levels of CD94/NKG2A and decreased KIR (Moroso, Metselaar et al. 2010).

In the uterus, NK cells play a critical role in pregnancy, as well as protecting the non-pregnant uterus against infection and malignancy. Uterine immune cell populations are mainly composed of macrophages and T cells but also include significant populations of NK cells (Bulmer and Lash 2005). uNK (uterine) cells are granular cytotoxic cells that are crucial in supporting angiogenesis during placentation (Chazara, Xiong et al. 2011). Phenotypically, uNK cells are CD56<sup>bright</sup>, CD16<sup>-</sup>, and CD94<sup>+</sup> and contain high numbers of cytolytic granules. Compared with PBNK cells, uNK cells include significantly higher proportions of (killer-immunoglobulin like receptors) KIR2D-double-positive cells that recognize non-classical HLA-C (human leukocyte antigen) molecules expressed by trophoblast cells (Male, Sharkey et al. 2011). In general, the uNKR repertoire does not differ significantly between pregnant and non-pregnant stages, with the exception of CD161, which is lower in dNK cells (so called during pregnancy), and CD69, shown to be lower in uNK cells (Male, Sharkey et al. 2011). dNK cells are potent producers of IFN- $\gamma$ , which is required for spiral artery remodelling during implantation (Moffett-King 2002). uNK cells are also prominent producers of tissue-remodelling factors, such as vascular endothelial growth factor C, placental growth factor, TGF- $\beta$ 1, Ang 1, and Ang 2 (Lash, Schiessl et al. 2006).

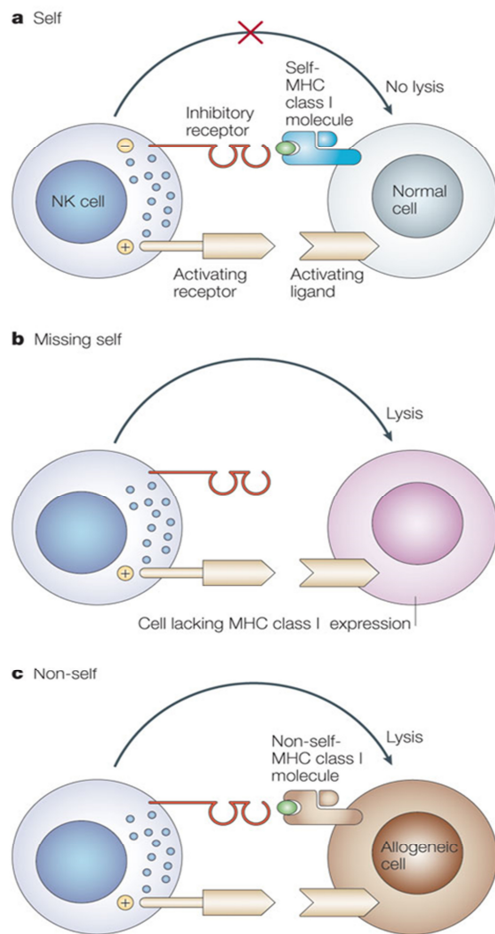
## 1.2 Target recognition by NK cells

Unlike B and T cells, NK cells belong to the innate immune system and do not express somatically rearranged receptors specific for antigens. NK cell-specific receptors are germ line-encoded and include a variety of activating and inhibitory receptors. The most significant receptors are the killer-immunoglobulin like receptors (KIR) that recognise human leukocyte antigen (HLA) class I proteins (HLA-A, -B and -C) (Vilches and Parham 2002). KIRs also play a pivotal role in the education process of NK cells where cells are primed to distinguish between self and non-self. Only after NK cells have contact with self-HLA molecules do they become functionally competent in a process called licensing (Fernandez, Treiner et al. 2005) (Kim, Poursine-Laurent et al. 2005). Individuals can have up to 15 KIR genes and the intracellular domain can either transmit an inhibitory or activating signal. Another inhibitory receptor expressed on NK cells is the heterodimer CD94/NKG2A with its counterpart NKG2C as an activating receptor, both recognizing HLA-E (Bryceson, March et al. 2006).

Most receptors bind their ligand through Ig domains, except for NKG2 homo- and heterodimers which have C-type lectin-binding domains. Inhibitory receptors have intracellular domains with multiple immune-receptor tyrosine-based inhibitory motifs (ITIMs) and many activating receptors possess intracellular domains containing immune-receptor tyrosine-based activation motifs (ITAMs). NKG2D and 2B4 receptors signal via tyrosine residues of the tyrosine-x-x-methionine (YxxM) and immune-receptor tyrosine-based switch motifs (ITSMs), respectively. DNAM-1 signalling involves phosphorylation of Ser329 by protein kinase C.

To contribute to the first line of defence, NK cells are poised ready to attack infected or malignant cells. This immediate response capacity of NK cells may present a

danger to healthy cells in the event of inappropriate NK cell activation, and consequently the process of NK cell activation is tightly regulated. Part of this regulation is inherent in the type of receptors that NK cells use to recognize and respond to target cells. Two suggested hypotheses of NK cell activation are the 'missing self' and 'induced self' theories as described in Figure 1.2 (Ljunggren and Karre 1990) (Watzl 2003).



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**Figure 1.2 Target cell recognition by NK cells.** (A) When the NK cell interacts with a normal (self) cell, activating signals might be triggered. However, the NK cell will not lyse the cell because it expresses self-MHC class I alleles. (B) Cells can lose the expression of MHC class I molecules because of viral infection or cancerous transformation. Consequently, the MHC-binding inhibitory receptor at the surface of the NK cell is not engaged and no inhibitory signals are transmitted. This process is known as the missing-self theory and results in killing of the target cell. (C) If the NK cell engages with allogeneic (non-self) cells, which express foreign MHC class I alleles, the target cell will be lysed because of insufficient inhibitory receptor binding (Kumar and McNerney 2005).

### **1.3 NK cell receptor repertoire**

#### **1.3.1 Activating and Inhibitory receptors**

NK cell activation programs result from the integration of multiple inhibitory (table 1.1) and activating (table 1.2) signals that vary depending on the nature of the interacting cells. These signals involve ITAM (immunoreceptor tyrosine-based activation motif)-bearing molecules and other stimulatory receptors and adhesion molecules, as well as (intracellular immunoreceptor tyrosine-based inhibitory motifs) ITIM. Inhibitory receptors signal through ITIM located in the cytoplasmic tail of these receptors. Commonly, Src homology 2 domain containing phosphatases (SHP1 or 2) are recruited after phosphorylation of a tyrosine residue (Tomasello, Blery et al. 2000). How inhibitory signals interfere with activating signals remains unclear, but recent reports suggest that ITIM-mediated signalling result in both dephosphorylation and specific phosphorylation of intracellular components. One recent report implicated b-arrestin 2 in the inhibition of NK cell activation, through the recruitment of SHP1 and SHP2 (Yu, Su et al. 2008). Another report suggested that a common point of NK cell activation signalling could be targeted, in which a SHP1 phosphorylation site (Vav1) could be de-phosphorylated during inhibitory signalling. This report showed that inhibitory signalling can prevent not only NK cell-mediated cytotoxicity, but also interfere with adhesion of NK cells to target cells (Bryceson, Ljunggren et al. 2009). In contrast, Long and Peterson (Peterson and Long 2008) have described the specific phosphorylation of a tyrosine adapter, Crk, as a result of ITIM engagement. Therefore, it is now clear that while ITIM signalling prevents intracellular phosphorylation, it is likely to involve more complex signalling than originally thought.

Receptor	Ligands
KIR2DL1	HLA-C2
KIR2DL2/3	HLA-C1
KIR2DL5	?
KIR3DL1	HLA-Bw4
KIR3DL2	HLA-A3, -A11
CD94-NKG2A	HLA-E
LILR	MHC class1, UL-18
LAIR1	Collagen
SIGLEC-3/-7/-9	Sialic acid
KLRG1	Cadherins
NKR-P1A (KLRB1)	CLEC2D (LLTI)

**Table 1.1 NK cell inhibitory receptors.**

Activating receptors on the cell surface of NK cells recognize ligands expressed on tumour-transformed or virally infected cells. The natural cytotoxicity receptors (NCR) bind to viral ligands, heat-shock associated proteins or tumour antigens and include NKp30, NKp44 and NKp46 (Pende, Parolini et al. 1999; Mandelboim, Lieberman et al. 2001; Baychelier, Sennepin et al. 2013). NKG2D is another essential activating receptor expressed on all NK cells and recognizes the major histocompatibility complex class I chain-related genes A and B (MICA and MICB) and UL16 binding protein family members 1-6 (ULBP1—6) (Cosman, Mullberg et al. 2001). Some

activating receptors signal through immunoreceptor tyrosine-based activating motifs (ITAMs), although these are not contained in the receptors' cytoplasmic tails but rather in associated molecules. After phosphorylation of a tyrosine residue in the tail, the Src homology 2 domain containing kinases (Syk or ZAP70) are recruited leading to a signal cascade which results in de-granulation and transcription of cytokine and chemokine genes (Tomasello, Blery et al. 2000).

Receptor	Ligands
<b>KIR2DL4</b>	HLA-G
<b>KIR2DS1</b>	HLA-C2
<b>KIR2DS2</b>	HLA-C1
<b>KIR2DS3</b>	unknown
<b>KIR2DS4</b>	unknown
<b>KIR2DS5</b>	unkown
<b>KIR3DS1</b>	HLA-Bw4
<b>CD94-NKG2C</b>	HLA-E
<b>CD94-NKG2D</b>	MIC-A/-B, ULBP 1/2/3/4
<b>CD94-NKG2E</b>	HLA-E
<b>CD160</b>	HLA-C

<b>NKp30</b>	BAT-3, HSPG, B7-H6, CMV-pp65
<b>NKp44</b>	Viral HA
<b>NKp46</b>	Viral HA, HSPG
<b>NKp80</b>	AICL
<b>DNAM-1</b>	PVR, CD122, Nectin 2
<b>2B4 act/inh</b>	CD48
<b>CD16</b>	IgG

**Table 1.2 NK cell activating receptors.**

A third activation/inhibition signalling pathway results from stimulation of the CD244 (2B4) receptor. This cytoplasmic tail of this receptor contains an immunoreceptor tyrosine-based switch motif, which recruit Src homology 2 domain containing adapter proteins SAP or ERT (Veillette 2006). Recruitment of SAP results in activation of NK cell function, wherein recruitment of ERT inhibits NK cell function.

A family of molecules that bind nectin and nectin-like proteins has recently emerged and have been shown to function as an important regulator of NK cell functions. These molecules include CD226, T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), CD96, and cytotoxic and regulatory T cell molecule (CRTAM).



### 1.3.2 DNAM-1 receptor

DNAM-1 receptor, also known as CD226, is a member of the Ig-superfamily and is constitutively expressed on approximately 50% of NK cells (Shibuya, Campbell et al. 1996). The ligands for this co-stimulatory activating receptor are CD155 (also referred to as Polio virus receptor, PVR or Necl-5) and CD112 (Nectin-2), and these ligands can be upregulated on some tumour cells, implicating DNAM-1 in some NK cell-mediated anti-tumour responses (Bottino, Castriconi et al. 2003) (Tahara-Hanaoka, Shibuya et al. 2004) (Masson, Jarry et al. 2001). In addition, DNAM-1 has been shown to be involved in the lysis of tumour cells that do not express ligands for NK cell-activating receptors, this therefore broadens the scope of tumours susceptible to NK cell-mediated responses, and suggests this receptor is more than just co-stimulatory (Gilfillan, Chan et al. 2008).

In humans, CD96 expression is confined chiefly to NK cells, CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells (Wang, O'Farrell et al. 1992). CD96 shares sequence similarity with CD226 and competes for binding to CD155 (Martinet and Smyth 2015). A recent study has found that CD96 competed with CD226 for CD155 binding and limited NK cell function by direct inhibition. As a result, CD96<sup>-/-</sup> mice displayed hyper-inflammatory responses to the bacterial product lipopolysaccharide (LPS) and resistance to carcinogenesis and experimental lung metastases. Their data provided the first description of the ability of CD96 to negatively control cytokine responses by NK cells (Chan, Martinet et al. 2014). Blocking CD96 may have applications in pathologies in which NK cells are important.

### **1.3.3 TIGIT**

It was recently shown that a new inhibitory receptor, named T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT), is expressed mainly on T cells and NK cells (Yu, Harden et al. 2009) (Boles, Vermi et al. 2009) (Stanietsky, Simic et al. 2009). Poliovirus receptor (PVR, also known as CD155) is identified as the physical ligand of TIGIT, and TIGIT/PVR engagement suppresses T-cell activation through regulation of IL-10 secretion by dendritic cells (Yu, Harden et al. 2009). TIGIT also has an intrinsic inhibitory function to T cells independent of antigen-presenting cells (Joller, Hafler et al. 2011) (Lozano, Dominguez-Villar et al. 2012). Additionally, the activation of TIGIT signaling can downregulate IFN- $\gamma$  secretion and cytotoxicity in both human and mouse NK cells (Stanietsky, Simic et al. 2009) (Stanietsky, Rovis et al. 2013). Recently, it was reported that TIGIT alleviates liver injury through negatively regulating NK-cell activation in murine acute viral hepatitis and also that TIGIT is a safeguard molecule to improve liver regeneration through negatively regulating NK–hepatocyte crosstalk (Bi, Zhang et al. 2014) (Bi, Zheng et al. 2014). Furthermore, regulatory T cells, also through expression of the co-inhibitory molecule TIGIT, selectively inhibit pro-inflammatory Th1 and Th17 cell responses (Joller, Lozano et al. 2014).

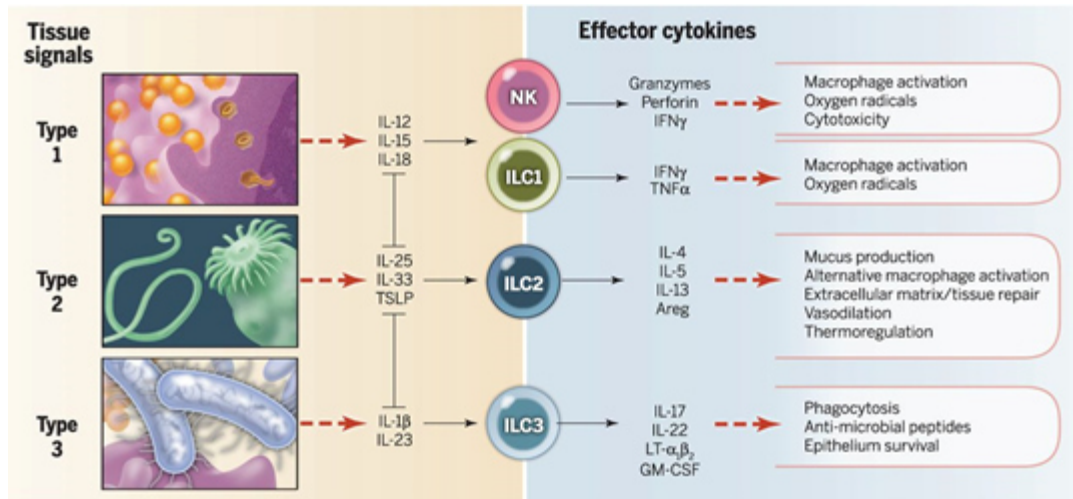
### **1.3.4 Cytotoxic and regulatory T cell molecule (CRTAM)**

CRTAM was discovered in 2000 using a cDNA library generated from NK and T cells (Kennedy, Vicari et al. 2000). The structure of CRTAM has some similarity to CD226 as it contains two extracellular immunoglobulin-like domains. Only one CRTAM ligand has so far been described, the nectin-like family member 2 (NECL2; also known as CADM1 and TSLC1). Unlike CD226 and CD96, CRTAM is not

expressed by resting NK cells but expression of this receptor is rapidly and transiently upregulated following NK cell activation (Arase, Takeuchi et al. 2005). CRTAM has been shown to enhance NK cell adhesion and cytotoxicity and to trigger the rejection of NECL2-expressing targets *in vivo* (Boles, Barchet et al. 2005). The generation of *Crtam*<sup>-/-</sup> mice revealed that CRTAM has a pivotal role in CD8<sup>+</sup> T cell retention within peripheral lymph nodes by favouring interactions with NECL2-expressing CD8α<sup>+</sup> dendritic cells (DCs) (Takeuchi, Itoh et al. 2009). CRTAM is also involved in stabilizing the conjugation between CD4<sup>+</sup> T cells and DCs. A recent study revealed that CRTAM interacts with the cell polarity regulator SCRIB to maintain T cell polarity after the early phase of T cell activation (Yeh, Sidhu et al. 2008).

#### **1.4 NK cell effector functions**

Conventional NK cells have been classified as members of the group 1 innate lymphoid cells (ILCs). Group 1 ILCs are defined by their ability to secrete IFN-γ in contrast to group 2 ILCs which secrete IL-13 and group 3 ILCs which produce IL-17 or IL-22 (Spits, Artis et al. 2013). ILCs are a growing family of immune cells that mirror the phenotypes and functions of T cells. NK cells can be considered the innate counterparts of cytotoxic CD8<sup>+</sup> T cells, whereas ILC1s, ILC2s, and ILC3s may represent the innate counterparts of CD4<sup>+</sup>Thelper1 (TH1), TH2, and TH17 cells. However, in contrast to T cells, ILCs do not express antigen receptors or undergo clonal selection and expansion when stimulated (Spits, Artis et al. 2013). Instead, ILCs react promptly to signals from infected or injured tissues and produce an array of secreted proteins, termed cytokines that direct the developing immune-response into one that is adapted to the original insult (Figure 1.3).



**Figure 1.3 Effector functions of innate lymphoid cells (ILCs).** (Eberl, Colonna et al. 2015).

Innate lymphoid cells (ILCs) differentiate from haematopoietic stem cells, via an ID2<sup>+</sup> precursor cell, under the influence of cytokines such as interleukin-7 (IL-7), IL-15, IL-23, IL-25 and IL-33. These signals induce the expression of transcription factors that promote the differentiation of the various ILC subsets (group 1, 2 and 3 ILCs) and induce their expression of signature cytokines. The cytokines secreted by ILCs promote important physiological responses, such as wound healing, tumour surveillance and protection against infections. However, ILC-derived cytokines can also promote immunopathology in diseases such as asthma and inflammatory bowel diseases (IBDs) (Walker, Barlow et al. 2013).

NK cells have four major effector functions: (1) the secretion of cytokines, (2) the release of perforins and granzyme, (3) killing through apoptotic pathways using FasL and TRAIL and antibody-dependent cellular cytotoxicity (ADCC) via the expression of Fc $\gamma$ RIIIA (CD16) on their cell surface.

#### **1.4.1 Secretion of cytokines**

An essential function of NK cells especially early in viral infections, is to release antiviral cytokines, such as IFN- $\gamma$  and TNF- $\alpha$  (Alter, Malenfant et al. 2004), as immune-defensive agents that additionally serve to activate resident inflammatory cells and recruit other cells (Biron, Nguyen et al. 1999). These NK cell-sourced cytokines also regulate dendritic cells, T cells, and B cells (Vivier, Tomasello et al. 2008) and regulate TNF-mediated apoptosis of the NK cells and neighbouring cells to delimit immune responses (Ross and Caligiuri 1997). Thus, cytokine production by NK cells influences both innate and adaptive immune responses (Degli-Esposti and Smyth 2005). Parenthetically, the uncontrolled release of TNF and other pro inflammatory cytokines in acute and chronic inflammatory disease is a major cause of ongoing tissue damage, pain, and fatality in these conditions (Bradley 2008). Uncontrolled cytokine release by NK cells and other cells together with defective granule release and target cell killing are concomitant features of hemophagocytosis and lymphohistocytosis in humans and in mouse models (Haddad, Wu et al. 2001) (Ishii, Ueda et al. 2005). Cytokine secretion is therefore central to immunity and to immunopathologies. Despite their importance, little is known about how cytokines are secreted by NK cells.

#### **1.4.2 Release of perforin and granzyme**

NK cells are renowned for their ability to kill virally infected or transformed host cells by release of cytotoxic granules containing granzymes and perforin. As a subset of cytotoxic lymphocytes, NK cells recognize and destroy malignant and virally infected cells (Cerwenka and Lanier 2001) (Lanier 2008). NK cells respond to signals generated by activating and inhibitory receptors on their surfaces to constrain killing

to appropriate target cells. Upon making contact with an appropriate target, NK cells release the membrane-disrupting protein, perforin, and proteolytic serine proteases, the granzymes, from secretory granules (Trapani and Smyth 2002).

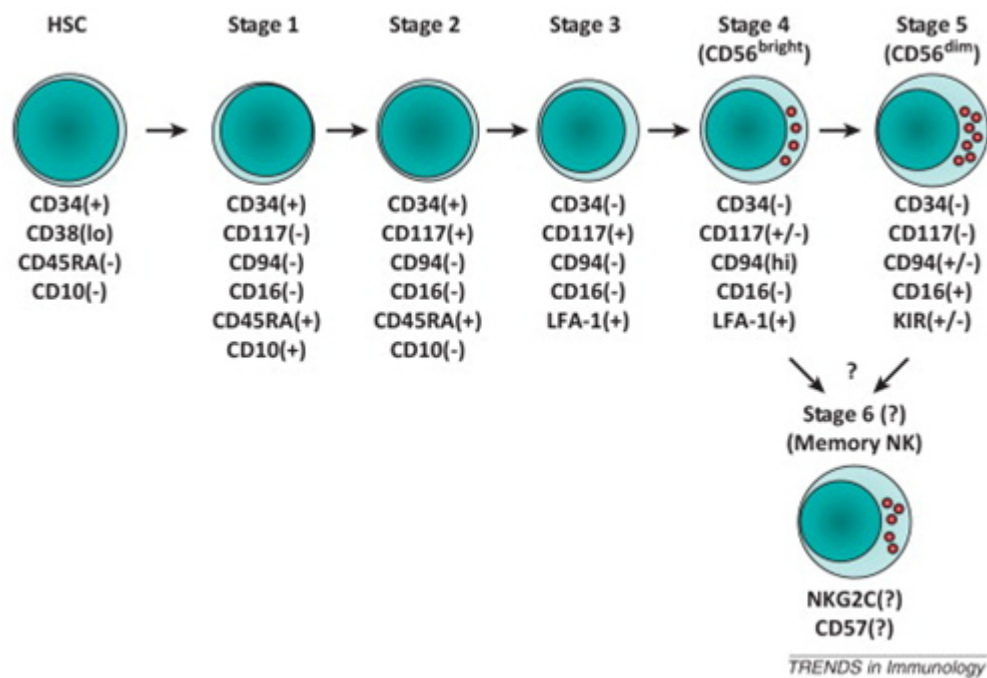
### **1.4.3 Killing through apoptotic pathways using FasL and TRAIL**

NK cells also use FasL and TRAIL to lyse different cells. FasL is constitutively expressed or induced upon interaction with target cells and induces  $\text{Ca}^{2+}$  independent, Fas (CD95/Apo1)-mediated apoptosis. Additionally, TRAIL transduces apoptotic signals upon binding their soluble ligand, the TNF-related apoptosis-inducing ligand (TRAIL). Fas ligand (FasL)-mediated cytotoxicity is initiated by NK cells through ligation of their activating receptors. FasL-mediated cytotoxicity is specifically directed against Fas- expressing target cells and is initiated by ligation of surface-bound homotrimeric FasL to the Fas receptor expressed on the target cell surface. This initiates the recruitment of “death domain” containing proteins, resulting in activation of the caspase cascade (Ashkenazi and Dixit 1998). The apoptotic cell presents with DNA fragmentation, nuclear membrane and cytoskeletal breakdown, plasma membrane blebbing, and the formation of membrane- enclosed apoptotic bodies, which are rapidly phagocytosed by neighbouring cells such as macrophages (Smyth and Trapani 1995) (Steller 1998). In addition, because cytotoxic lymphocytes, including NK cells, express Fas, the Fas/FasL apoptotic pathway may also play an important role in downregulating the immune response. This occurs by inducing FasL-mediated apoptosis in NK cells after the upregulation of FasL, leading to activation induced cell death (AICD) (Lynch, Ramsdell et al. 1995). The upregulation of both FasL mRNA expression and cytotoxicity have been shown to involve activation through the CD16 (FcγRIIIA) receptor in the presence of interleukin IL-2 stimulation (Ortaldo, Mason et al. 1995).

TRAIL, was first described as a cytokine capable of inducing apoptosis in a wide variety of cancer cells while sparing normal cells. However, its main role seems to be regulation of the immune response (Corazza, Brumatti et al. 2004). The role of endogenous TRAIL in tumour immune surveillance is not fully understood yet. A few *in vitro* studies have clearly shown that NK cells are able to kill cancer cells using TRAIL (Takeda, Smyth et al. 2001) (Cretney, Takeda et al. 2002).

## **1.5 Human NK Cell Development**

NK cells have a short half-life of less than 10 days. They are continuously replenished through differentiation from haematopoietic stem cells in the bone marrow (BM) and specific subtypes reside in the liver, uterus and to lesser extent in the lymph nodes and thymus (Yu, Freud et al. 2013). NK cells primarily develop in the bone marrow, thymus and lymph nodes and the resident and recruited NK cells play an important role in immunosurveillance and homeostasis of mucosal surfaces (Vivier, Tomasello et al. 2008; Colonna 2009). The development of NK cells occurs in a series of maturation steps that result in the acquisition of specific functionality. The schematic representation of human NK cell development is summarised in Figure 1.4.



**Figure 1.4 Schematic overview of human NK cell development.** Memory NK cells are displayed as a possible terminal stage of maturation (stage 6). (Yu, Freud et al. 2013).

Human NK cells develop from BM-derived HSCs via a common lymphoid progenitor which has the potential to differentiate into all lymphocyte lineages (Blom and Spits 2006). Human HSCs are defined as CD34<sup>+</sup>CD38<sup>dim/-</sup>CD45RA<sup>-</sup>CD10<sup>-</sup> and CLPs as CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup>CD10<sup>+</sup> within the BM-derived haematopoietic progenitor cell (HPC) pool (Galy, Travis et al. 1995). An important step in human NK cell development is the acquisition of IL-15 receptor  $\beta$  chain (CD122). IL-15 promotes NK cell development, maturation and survival (Becknell and Caligiuri 2005). Both stage 2 and 3 precursor cells respond to IL-15 and can give rise to mature NK cells *in vitro*. In the late stages of NK cell development, acquisition of functional receptors occurs. CD56, CD94, NKp46 and NKG2D are upregulated in the first maturation steps, followed by KIRs and CD16 (Freud and Caligiuri 2006) (Grzywacz, Kataria et



al. 2006). Mature NK cell subsets (stage 4 and 5) differ in their surface marker expression and functionality and have been characterised as CD56<sup>dim</sup> and CD56<sup>bright</sup> (Cooper, Fehniger et al. 2001). Studies suggest that CD56<sup>bright</sup> NK cells are precursors to CD56<sup>dim</sup> NK cells (Caligiuri 2008) (Huntington, Legrand et al. 2009). CD56<sup>bright</sup> NK cells are more prevalent in umbilical cord blood and neonatal tissues whereas the CD56<sup>dim</sup> NK cell population increases later in life and exhibits shorter telomeres (Romagnani, Juelke et al. 2007). Although NK cells are classified as cells of the innate immune system, it is believed that they can exhibit memory function. In humans, memory NK cells could be described as the final stage of NK cell maturation, although the origin within the stages of NK cell differentiation is not clear yet (Romee, Schneider et al. 2012). Recently it has been demonstrated that mature NK cells possess characteristics of immunological memory, including antigen-specific response - similar to B and T cells – and antigen-independent memory induced by exposure to a specific cytokine milieu. Antigen-specific NK cell memory was first demonstrated in the setting of hapten-induced contact hypersensitivity in RAG2-deficient mice, lacking B and T cells (O'Leary, Goodarzi et al. 2006) (Paust, Gill et al. 2010). In these studies it was shown that NK cells were responsible for the secondary response and that a subset of hepatic NK cells mediated the response. Importantly, antigen-specificity of NK cells has further been demonstrated for the germline-encoded activating receptor Ly49H, which uniquely recognises the MCMV-encoded glycoprotein m157 and receptor-ligand interaction results in the activation and proliferation of Ly49H<sup>+</sup> NK cells (Arase, Mocarski et al. 2002) (Smith, Heusel et al. 2002) (Brown, Dokun et al. 2001). The adoptive transfer of Ly49H<sup>+</sup> NK cells into mice lacking the receptor revealed expansion of this specific NK cell subset within 1 week following MCMV infection and a pool of memory NK cells persisted in both lymphoid and non-lymphoid tissues several months after infection (Sun, Beilke et al. 2009). Although the evidence supporting the generation of NK cell memory is

continuously growing, a lot of questions regarding mechanisms remain unanswered. Addressing questions about involved receptors/ligands, influence of epigenetic setup or if memory NK cells remain tissue-resident or continue circulating will help to utilize NK cells for successful immunotherapy.

### **1.5.1 Transcriptional control of ILC development**

The commitment of the NK cell lineage and further maturation requires the expression of a range of specific transcription factors (TFs) including PU.1, T-bet, Ets-1, TOX and E4BP4 (also known as NFIL3) (Colucci, Samson et al. 2001; Gascoyne, Long et al. 2009; Aliahmad, de la Torre et al. 2010; Gordon, Chaix et al. 2012; Ramirez, Chandler et al. 2012). The transcriptional network that regulates differentiation of distinct ILC groups from a common CLP progenitor is summarised in figure 1.5. NK cells, including conventional NK (cNK) and thymic NK (tNK) cells, are characterised as cytotoxic ILC1 and are defined by IFN $\gamma$  production and their requirement of T-bet and Eomes TFs (Klose, Flach et al. 2014) (Gordon, Chaix et al. 2012).



expression of E4bp4 and it is suggested that E4bp4 acts both up- and downstream of IL-15, forming a feedback loop that drives commitment to the NK cell lineage (Male, Nisoli et al. 2014). T-bet and Eomes act at later stages in NK cell development and promote cell maturation and function (Gordon, Chaix et al. 2012) (Seillet, Huntington et al. 2014).

Furthermore, the cytokine milieu has a significant role in NK cell development. IL-15 is pivotal not only for NK cell survival and proliferation, but also for NK cell differentiation, in combination with Flt3L, SCF and IL-7 (Freud and Caligiuri 2006). IL-21 has been shown to regulate KIR expression in later stages of NK cell development (Sivori, Cantoni et al. 2003).

## **1.6 NK cells and Immunotherapy**

First immunotherapy studies with NK cells focused on the activation and expansion of autologous NK cells. A study from 2011 (Parkhurst, Riley et al. 2011) used autologous PBMC, depleted of CD3 T lymphocytes, and expanded them on a feeder layer of autologous PBMC in the presence of IL-2. Eight patients with renal cancer or advanced melanoma received an average of  $4.7 \times 10^{10}$  ( $\pm 2.1 \times 10^{10}$ ) NK cells after chemotherapy with Cytosan/Fludarabine, followed by administration of IL-2 (720,000 IU/kg every 8h). In this study no clinical responses were detected, but the transferred NK cells stayed present in the PB and were able to mediate ADCC with rituximab or anti HER2/neu antibodies. The important conclusion of this study was that even treatment with lympho-depleting chemotherapy, extensive *ex vivo* NK cell expansion and high doses of IL-2 will not necessarily result in a clinically relevant anti-tumour response. In general, information from this and other studies resulted in the following important findings: IL-2 can be administered to patients in a

safe manner daily up to 3 times/week and it increases the level of circulating lymphocytes with significant higher NK cells numbers.

Further research revealed that autologous NK cells are unlikely to kill tumours because of the inhibitory signals resulting from KIR-self-MHC interaction. These findings led to the use of allogeneic NK cell for immunotherapies and several studies have since been conducted. Miller et al. (Miller, Soignier et al. 2005) infused patients with renal cell carcinoma or leukemia (prior treatment with cyclophosphamide and fludarabine) with CD3-depleted lymphocytes containing 20-42% CD56<sup>+</sup> NK cells from an MHC haplotype-mismatched relative, followed by IL-2 injections ( $1.75 \times 10^6$  IU/m<sup>2</sup> for 3 weeks or 10 MU 3 times per week). They observed that 5 out of 19 patients with acute myeloid leukemia (AML) obtained a remission, but no response was detected in patients with renal cancer which lead to the assertion that response to NK cell infusion might be dependent on disease. The infusions were well tolerated except for occasional fever and rigors. Another study treated 10 children with good/intermediate prognosis AML with KIR-HLA-mismatched allogeneic NK cell infusions (CD3 depleted), after front line chemotherapy (Rubnitz, Inaba et al. 2010). The NK cell infusions were well tolerated and NK cells could be detected in all patients after infusion, with 3 children having cells detected up to 1 month. The promising outcome of this study was that all patients remained in remission for at least 2 years. Although the study included only a small cohort of patients, it suggests that KIR-HLA mismatching is beneficial. In another study from Curti et al. (Curti, Ruggeri et al. 2011), older AML patients were transfused with allogeneic KIR-mismatched NK cells after treatment with cyclophosphamide and fludarabine (Cy/Flu). Three of six patients were in complete remission and remained disease-free at 34,32 and 18 months. One patient had a transient response only. Recent results of clinical studies are summarised in table 1.3.

NK cell product	Patient characteristics	Outcome	Reference
<b>NK cell line NK92:</b> Irradiated NK92 cells, doses of $1 \times 10^8$ – $3 \times 10^9$ cells/m <sup>2</sup> .	11 adult patients (10 renal cell carcinoma and 1 melanoma).	Infusion complicated with some febrile reactions. Two patients with possible tumor responses.	Arai et al. [63]
Irradiated NK92 cells, doses of $1 \times 10^9$ up to $1 \times 10^{10}$ cells/m <sup>2</sup>	13 patients with solid tumors (7 sarcomas, 2 patients with leukemia/lymphoma).	No severe side effects, response in one patient with advanced lung cancer.	Tonn et al. [37]
<b>Autologous NK cells:</b> PBMNC derived, stimulated with IL-2 and $\alpha$ -galactosylceramide, doses: $1 \times 10^7$ – $5 \times 10^7$ cells/m <sup>2</sup> administered at 2 and 3 weeks after apheresis.	Six patients with non-small cell lung cancer (all adult).	Infusion complicated with febrile reactions, transient arrhythmia, headache. No tumor responses.	Motohashi et al. [73]
PBMNC derived, stimulated with IL-2 and a Hsp70 derived peptide, doses: $1 \times 10^6$ to $7.5 \times 10^6$ .	12 patients with colon or lung carcinoma (all adult).	Infusion complicated by itching in one patient. No significant tumor responses.	Krause et al. [74]
PBMNC derived; CD3 <sup>+</sup> depleted; stimulated with irradiated autologous PBMNCs as feeder cells, IL-2, and OKT3, doses: $1.88 \times 10^{10}$ to $7.6 \times 10^{10}$ NK cells.	Eight patients with melanoma or renal cell carcinoma (all adult).	Infusions complicated by shortness of breath in one patient, otherwise no reactions. No tumor response.	Parkhurst et al. [58]
PBMNC CD3 <sup>+</sup> depleted, CD56 <sup>+</sup> enriched, stimulated with irradiated feeder cell line EBV-TM-LCL and IL-2, doses: up to $1.88 \times 10^9$ /kg.	n = 14 patients (CLL and solid tumors).	Thyroiditis and constitutional symptoms.	Lundqvist et al. [61]
<b>Allogeneic NK cells:</b> Related allogeneic NK cells (CD34 <sup>+</sup> progenitor cells from donor were expanded and differentiated into NK cells with IL-15, IL-21, and hydrocortisone) at doses of $1.8 \times 10^8$ to $6.3 \times 10^8$ cells administered 43–50 d after allogeneic SCT.	14 patients with AML, ALL, or high-grade MDS (all adult).	No infusion-related complications. Two patients with active leukemia had no benefit.	Yoon et al. [75]
Related allogeneic NK cells (PBMNC derived, stimulated with IL-2 and OKT3) at doses of $1 \times 10^6$ to $10 \times 10^6$ cells/kg, administered at 13 to 41 d after autologous SCT.	Six patients with colon carcinoma, hepatocellular carcinoma, renal cell carcinoma, or chronic lymphocytic lymphoma (all adult).	Infusions complicated by febrile reactions, nausea, coughing, hemoptysis, and melena. Patient with hepatocellular carcinoma had stable disease, otherwise no tumor response.	Barkholt et al. [76]
Haploidentical allogeneic NK cells (PBMNC derived, CD3 <sup>+</sup> depleted, CD56 <sup>+</sup> enriched, IL-2 stimulated); doses up to $30 \times 10^6$ /kg post SC.	Two pediatric patients with high risk ALL and one patient with AML, all haploSCT in blast persistence.	All three patients reached remission for several weeks/ months, two patients died during the next relapse, one due to infection.	Koehl et al. [77]
Haploidentical allogeneic NK cells (PBMNC derived, CD3 <sup>+</sup> depleted, CD56 <sup>+</sup> enriched, IL-2 stimulated); doses: $6 \times 10^6$ to $45.1 \times 10^6$ cells/kg on days (+3), 40 and 100 post haploSCT.	Four patients with neuroblastoma, four with AML, one with ALL (all pediatric and mostly non in remission at haploSCT).	Infusion complicated with some febrile reactions and two patients with vomiting and blood pressure changes. Two patients with high-risk neuroblastoma alive at 2 y.	Brehm et al. [8]
Haploidentical allogeneic NK cells (PBMNC derived, CD3 <sup>+</sup> depleted, stimulated with IL-2 overnight) at doses of $8.33 \times 10^6$ to $3.94 \times 10^7$ cells/kg.	20 patients with breast or ovarian carcinoma (all adult).	Infusions complicated by dyspnea, hypoxia, febrile reactions, hypertension, hypotension, fatigue, edema, pneumonitis, rash, nausea, myalgia. Two patients developed passenger lymphocyte syndrome.	Geller et al. [27]
PBMNC derived (haploidentical and autologous NK cells) activated/ expanded with K562, genetically modified to express 41BB-ligand and membrane-bound interleukin (IL)15; doses: up to $1 \times 10^9$ /kg.	Eight patients high risk myeloma.	Seven patients without side effects, one patients showed 7 d after cell infusion that donor NK cells comprised >90% of circulating leukocytes.	Szmania et al. [78]
Donor CD56 <sup>+</sup> NK cells were cultured for 20–23 d with interleukin-15 and hydrocortisone; doses: $0.2$ – $29 \times 10^7$ cells/kg.	16 patients with adenocarcinoma or squamous cell carcinoma.	No severe side effects, two patients with partial response and six patients with disease stabilization.	Iliopoulou et al. [79]
Donor-derived IL-15/4–1BBL-activated NK cells following HLA-matched, T-cell-depleted SCT, doses: $1$ – $10 \times 10^6$ cells/kg.	Nine pediatric patients and young adults with high risk solid tumors.	5/9 patients developed GvHD grade III/IV.	Shah et al. [80]

**Table 1.3 Overview of recent NK cell clinical trials with in vitro activated NK cells. (Koehl, Kalberer et al. 2016)**

### **1.6.1 Challenges of NK cell immunotherapy**

So far, adoptive NK cell therapy has shown promising results in the treatment of haematological diseases, mainly in AML patients, but treatment of other cancers has been challenging and there has not yet been a clinical breakthrough for NK cell therapy. The obstacles for successful NK cell immunotherapy include low NK cell expansion and activation status and the risk of graft versus host disease (GVHD) by residual T- or B cells. Furthermore, the suppressive milieu within the tumour environment and the activation of suppressive T lymphocytes (Tregs) followed by IL-2 administration has been shown to suppress NK cell activity (Sim, Martin-Orozco et al. 2014). Methods therefore have to be found to (1) improve the anti-tumour cytolytic properties of the derived NK cells and (2) to generate large numbers of NK cells to promote clinical efficacy.

### **1.6.2 NK cell engineering**

To enhance the anti-tumour activity of lymphocytes and to direct them specifically to tumour cells, transgene chimeric antigen receptors (CAR) can be expressed on their cell surface. This approach has shown great promise in T cell-therapies against CLL cells (Grupp, Kalos et al. 2013). Although the focus currently lies on CAR-transduced T cell therapy, NK cells could be useful as an alternative lymphocyte source for CAR-directed therapies. The disadvantage of NK cells is the low number in PBMCs that can be isolated and low transfection and expansion efficiencies. However, a number of studies have used the NK cell line, NK-92, transduced with CARs directed towards CS1-expressing multiple myeloma (MM) cells (Chu, Deng et al. 2014), CD19 and CD20 (Boissel, Betancur-Boissel et al. 2013). Another report

shows the expression of the chimeric receptor CD4ζ in hiPSC-derived NK cells targeting HIV-infected cells (Ni, Knorr et al. 2014).

### **1.6.3 NK cell sources for immunotherapy**

#### **1.6.3.1 Peripheral blood-derived NK cells**

NK cells for immunotherapy can be derived from various sources. Because large numbers of functional NK cells are needed for adoptive cell transfer, several expansion methods have been described in the literature using either cytokines or artificial antigen-presenting cells (aAPCs) (Cheng, Chen et al. 2013; Shah, Martin-Antonio et al. 2013). Further sources are the NK cell line NK-92, which is an IL-2 dependent cell line derived from a 50 year old male with rapidly progressive non-Hodgkin's lymphoma (Gong, Maki et al. 1994).

#### **1.6.3.2 Cord blood-derived NK cells**

The differentiation of NK cells from haematopoietic stem cells is an alluring option. High numbers of clinically appropriate NK cells can be generated from either umbilical cord blood (UCB) or mobilized PB-CD34<sup>+</sup> cells in cytokine-dependent differentiation protocols (Grzywacz, Kataria et al. 2006; Spanholtz, Preijers et al. 2011). Derived NK cells show high expression of activating NK cell receptors and cytolytic activity against K562 cells. Stromal cell-based *in vitro* differentiation methods include the co-culture on mouse fetal liver cell lines EL08.1D2 or AFT024 and cells are cultured for a period of 4-6 weeks (Pinho, Marques et al. 2012; Luevano, Domogala et al. 2014).

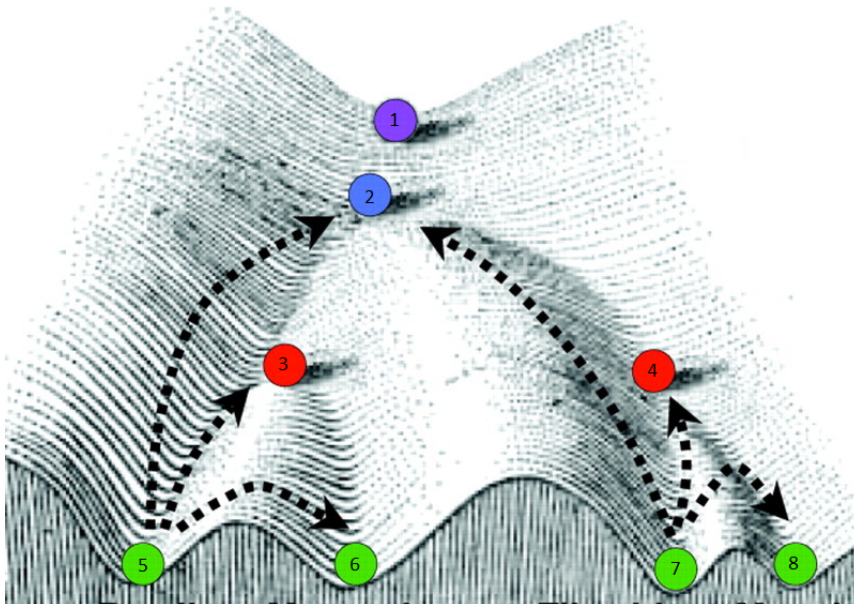


#### 1.6.3.3 HPSC-derived NK cells

The *in vitro* derivation of large numbers of functional lymphocytes from hPSC sources has huge potential for immunotherapy. In contrast to umbilical cord blood HSCs, hiPSCs can be expanded indefinitely whilst remaining their pluripotent characteristics. This further provides a platform for genetically modifying cells before differentiating them into the lineage of interest. In contrast, the *ex vivo* manipulation of UCB-derived CD34<sup>+</sup> cells is less efficient and limited. In addition, NK cell therapy with UCB-derived NK cells is dependent on cord blood donations with the desired tissue type. NK cells generated from hPSCs could overcome this limitation by establishing a cell database covering all MHC classes and providing an off-the-shelf cell therapy.

## 1.7 Stem cells

Mammalian development starts with the fertilization of an oocyte with a spermatocyte. This fusion results in a cell called zygote, which undergoes numerous cell divisions to give rise to any specialised cell type within the organism. The process of cell type specialisation is called differentiation. The Waddington model describes the developmental potential of differentiating cells with the metaphor of a ball rolling down a mountain (Figure 1.6).



**Figure 1.6 The developmental potential and epigenetic states of cells at different stages of development.** (Hochedlinger and Plath 2009) A modification of C. H. Waddington's epigenetic landscape model, showing cell populations with different developmental potentials (left) and their respective epigenetic states (right). Developmental restrictions can be illustrated as marbles rolling down a landscape into one of several valleys (cell fates). Coloured marbles correspond to different differentiation states (purple, totipotent; blue, pluripotent; red, multipotent; green, differentiated).

unipotent). Examples of reprogramming processes are shown by dashed arrows. Adapted from (Hochedlinger and Plath 2009).

The ball in the above figure represents a stem cell which rolls down a mountain and can take different routes, which represent different lineages during differentiation. At the very top of the mountain, the ball has the opportunity to take any route downhill. The further the ball rolls down a specific route, the more difficult it becomes to go back uphill or cross to a different route. This model represents the differentiation potential of stem cells at different stages in development: totipotent, pluripotent and multipotent.

Totipotent stem cells are defined as cells that can give rise to a whole organism – including both, embryo proper and extraembryonic tissue. After numerous cell divisions, the blastocyst is formed at day five of human development. Cells of the inner cell mass (ICM) at this stage are defined as pluripotent stem cells (PSCs), which have the ability to differentiate into cells of all three germ layers including ectoderm, mesoderm and endoderm. As cell divisions continue during early development, cells become more specialized and less plastic. Multipotent stem cells can give rise to a certain lineage of cell types – for example haematopoietic stem cells, from which the whole haematopoietic system within an organism is formed.

### **1.7.1 Embryonic stem cells**

In 1998 the first human PSCs, embryonic stem cells (ESCs), were derived from cells of the inner cell mass of early stage blastocysts (Thomson, Itskovitz-Eldor et al. 1998). Since then, a variety of protocols has been established showing the differentiation potential of these cells to all three lineages including cardiomyocytes, neurons and haematopoietic progenitors (Lerou and Daley 2005).

Human ES cells have major clinical potential in tissue repair, disease modelling and drug screening and the interest in hESCs for regenerative medicine research increased significantly. However, the use of hESCs is highly controversial because cells are derived from human pre-implantation embryos. Most hESC lines are established from spare embryos from *in vitro* fertilisation (IVF) but the production of embryos specifically for therapeutic cloning via somatic cell nuclear transfer is under discussion. This procedure aims to derive hESCs – genetically identical to the patient - by transfer of a patient's somatic cell-nucleus to an unfertilized egg. These cells could be used as an unlimited cell source for a variety of somatic cells for autologous cell transplantation (de Wert and Mummery 2003). The moral aspects of hESC research, regarding the conditions and restrictions of isolation and use, have to be considered by researchers and are subject to governmental regulations. Laws for governing stem cell research vary in different countries, with the UK being the first to pass a law. The European Science Foundation reports on the scientific, ethical and legal issues in human stem cell research within the European Union.

### **1.7.2 Induced pluripotent stem cells**

To overcome the ethical and logistical issues related to hESCs, research focussed on identifying factors required for maintaining the pluripotent state of cells. In 2006 a major breakthrough was achieved when the first induced pluripotent stem cells (iPSCs) were generated by Kazutoshi Takahashi and Shinya Yamanaka who discovered the crucial role of the core transcriptional network of pluripotency in murine cells (Takahashi and Yamanaka 2006). This study showed that the over-expression of the four transcription factors Oct4, Sox2, Klf4 and c-Myc by retrovirus-mediated transduction in murine fibroblasts generated cells with most properties of PSCs (Takahashi and Yamanaka 2006). One year later, human iPSCs (hiPSCs)

were obtained by using the same four transcription factors (Takahashi, Tanabe et al. 2007).

### **1.7.3 Reprogramming methods**

Since the use of retrovirus for generation of first induced pluripotent stem cells, many other methods have been generated and they can be divided into two categories: integrative and integration-free. Integrative methods include retrovirus and lentivirus and using this system the transgenes are integrated in the host genome of the cells. The advantage of integrative system is that the reprogramming efficiency is much higher. Due to the integration of transgenes into the host system this can potentially affect the genetic stability of the cells and poses a risk for tumourigenesis as some of the reprogramming factors are oncogenes such as c-myc.

Non-integrative methods include use of Sendai virus, RNA, protein and episomal plasmid based methods. Sendai virus has the advantageous property that it is an RNA virus that does not enter the nucleus and is therefore diluted out of cells after infection. Cells are reprogrammed in ~25 days at an efficiency of 0.1% for blood cells and 1% for fibroblasts. One disadvantage of Sendai-based reprogramming is that it takes ~10 passages for the virus to be completely lost from recently reprogrammed iPSCs and cells may need to be cultured at a higher temperature (39°C) to inactivate the virus.

The ability to express reprogramming factors as mRNA offers another method to make integration-free iPSCs (Warren, Manos et al. 2010). Warren and colleagues were able to reprogram human fibroblasts at an efficiency of 1.4% within 20 days and the efficiency increased to 4.4% in hypoxic conditions. Although reprogramming

factor mRNAs are commercially available, this method suffers from the fact that it is labour intensive, requires addition of mRNA daily for 7 days, and has not been validated in cells other than fibroblasts.

Expression of reprogramming factors as proteins would be an ideal method to generate integration-free iPSCs. Unfortunately, it has been technically challenging to synthesize large amounts of bioactive proteins that can cross the plasma membrane. The low efficiency, technical challenges, and lack of published studies in non-fibroblast cell types suggest that much work needs to be done before protein reprogramming is a viable method (Kim, Kim et al. 2009). Transient expression of reprogramming factors as episomal plasmids would allow for the generation of footprint-free iPSCs. Yu et al. (Yu, Vodyanik et al. 2007) used oriP/Epstein-Barr nuclear antigen-1 based episomal vectors (oriP/EBNA1) with an overall low reprogramming efficiency. Okita et al. (Okita, Matsumura et al. 2011) improved the reprogramming efficiency by introducing small hairpin p53 suggesting a link between reprogramming efficiency and tumour generation (Marion, Strati et al. 2009). In addition C-Myc was replaced with L-Myc. In our lab a systematic evaluation of different methods for generating hiPSCs was performed with the aim of developing a feeder- and xeno-free method suitable for scale up and clinical translation (Goh, Caxaria et al. 2013). The reprogramming efficiency of three different primary fibroblast lines - two derived from patients with rapid onset Parkinsonism dystonia and one from an elderly healthy volunteer - was 4 fold higher with the episomal plasmid method when compared to the retroviral method and over 50 fold higher than the mRNA method. Additionally, with the plasmid reprogramming protocol, commercially available, fully defined, xeno-free media and supplements could be used, without significantly impacting the reprogramming efficiency. The data suggests that the non-viral, episomal plasmid method is most efficient and

robust at reprogramming patient-derived human fibroblasts and it is currently transferred into a GMP environment for clinical use.

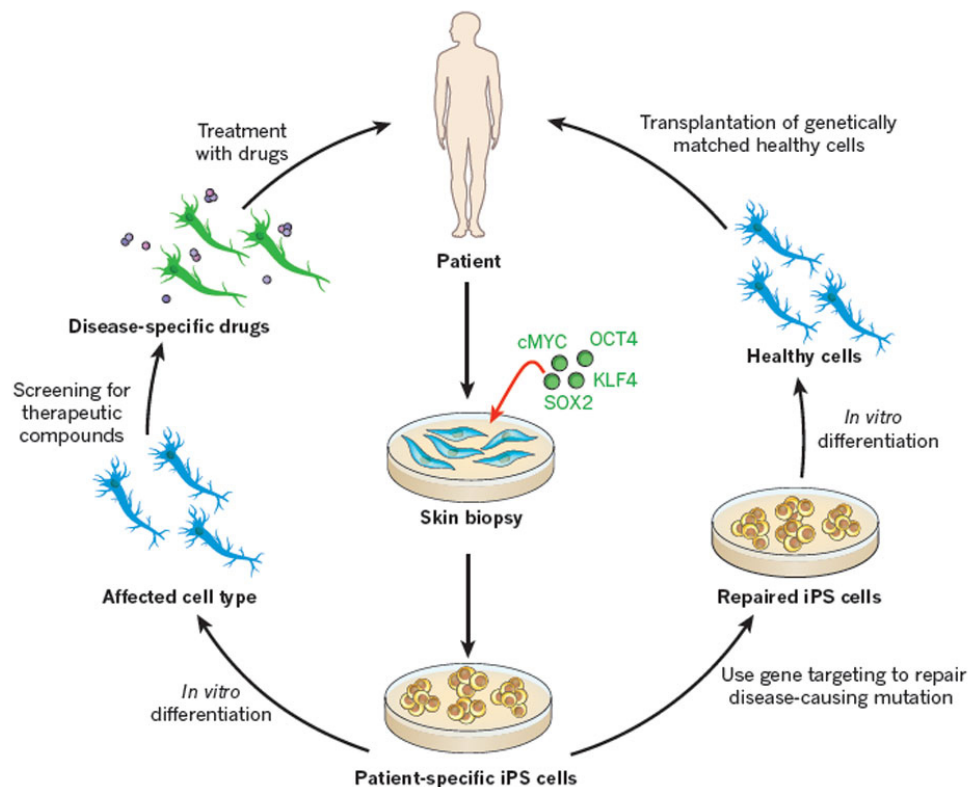
#### **1.7.4 Reprogramming cocktail**

Since the initial report of reprogramming, different combinations of factors have been used to reprogram somatic cells. Thomson et al. tested whether Oct4, Sox2, Nanog and Lin-28 are sufficient to induce pluripotency and showed successful reprogramming of human fibroblasts without c-Myc (Yu, Vodyanik et al. 2007). A potential tumour risk associated with some of the reprogramming factors - Lin-28 and c-Myc – is a main challenge for clinical applications of hiPSC technology. Other studies replaced some of the transcription factors with chemicals and/or small molecules such as valproic acid and RepSox to improve the reprogramming efficiency (Huangfu, Osafune et al. 2008; Shi, Do et al. 2008). Furthermore, it has been demonstrated that the somatic cell type can greatly influence the efficiency of iPSC derivation and it was suggested that the subsequent differentiation of these cells can be influenced by their epigenetic memory (Kim, Doi et al. 2010).

#### **1.7.5 Clinical application**

Overall, human iPSCs have gained incredible interest in regenerative medicine as they offer an indefinite supply of patient-specific cells that can be differentiated into any somatic cell type, offering immunologically compatible cells for transplantation and cell replacement therapies (Figure 1.7). Many differentiation studies have been conducted to gain better insight into the kinetics, characteristics and potential of differentiated cell types. In addition to conventional differentiation approaches,

recent studies showed that the over-expression of cell-specific TFs can change the cell identity of somatic cells in a process termed direct lineage-conversion or transdifferentiation which bypasses the intermediate pluripotent state (Pereira, Chang et al. 2013). This principle has been shown with various somatic cell types including human fibroblasts to multi-lineage blood progenitors (Szabo, Rampalli et al. 2010) and Alzheimer's disease fibroblasts into neurons (Qiang, Fujita et al. 2011). These findings demonstrate an alternative approach to reprogramming to the pluripotent state, but also sacrifice the unlimited cell source provided by pluripotent stem cells.



**Figure 1.7 Application of hiPSC technology.** HiPSCs have the potential to be used to model and treat human disease. Patient-specific iPSCs can be derived by ectopic co-expression of transcription factors in cells isolated from a skin biopsy can



be used in one of two pathways. In cases in which the disease-causing mutation is known gene targeting could be used to repair the DNA sequence (right). The gene-corrected patient-specific iPSCs would then undergo directed differentiation into the affected neuronal subtype and be transplanted into the patient's brain. Alternatively, directed differentiation of the patient-specific iPSCs will allow the patient's disease to be *modelled in vitro*, and potential drugs can be screened, aiding in the discovery of novel therapeutic compounds (Robinton and Daley 2012).

Several clinical trials are underway utilising embryonic stem cells as well as induced pluripotent stem cells in regenerative medicine. HPSC – based therapy moved into clinic surprisingly quickly and only twelve years after the first hPSCs have been isolated, the first patient has been treated with hESC-derived cells. There are several clinical trials (table 1.4) ongoing and their outcome will give valuable information about the safety and efficacy of PSC-based therapies.

Disease type	Cell source	Institution	Country	Start date	Finish date	Subject
Spinal cord injury	hESC	Geron	USA	Oct 2010	July 2013	5
		Asterias	USA	March 2015	June 2018	13
Immunotherapy vaccine for lung cancer	hESC	Asterias	UK	Not defined	Not defined	Not defined
Geographic atrophy secondary to myopic macular degeneration	hESC	Ocata	USA	April 2014	April 2015	Not defined
Stargardt macular degeneration of	hESC	Ocata	USA	July 2012	Dec 2030	13

retina			UK	Nov 2011	Dec 2015	16
Dry macular degeneration of retina	hESC	Ocata Cell cure Neurosc.	USA	July 2012	Dec 2030	13
			Israel	April 2015	Aug 2017	15
	hiPSC	RIKEN CBD	Japan	Oct 2013	Not defined	6
Wet macular degeneration of retina	hESC	The London Project to cure blindness	UK	Aug 2015	Oct 2016	3
Dry macular degeneration	hiPSC	The London Project to cure blindness	UK	April 2016	Not defined	3
Diabetes type I	hESC	ViaCyte	USA	Sep 2014	Aug 2017	40
Heart failure	hESC	APHP	France	June 2013	June 2017	6

**Table 1.4 Clinical trials with hPSCs.** (Ilic, Devito et al. 2015)

### 1.7.6 Challenges of hiPSC based therapy

Despite the great enthusiasm about the potential of hPSCs, there are still many challenges that need to be addressed. One big issue is the ability of hPSCs to induce tumour formation within the recipient. Consequently, it is essential to verify that no cells with pluripotent potential are present in hiPSC-derived engraftments. Contradictory reports are published regarding the immunogenicity of patient-specific

hiPSCs. Zhao et al. (Zhao, Zhang et al. 2011) reported in their study that teratomas – formed from “self” hiPSCs – which triggered an immune response in mice. This result was unexpected because it was believed that these cells would not cause any immune response due to self-tolerance and triggered a debate on safe use of hiPS-derived cells. However, two recent reports on using *in vitro* differentiated cells from syngeneic hiPSCs showed no evidence of immune rejection of tissue derived from either hESCs or hiPSCs (Araki, Uda et al. 2013; Guha, Morgan et al. 2013). In a further study hESCs have been differentiated into retinal pigment epithelium (RPE) and these cells transplanted into patients with Stargardt's macular dystrophy and dry age-related macular degeneration - the leading cause of blindness in developed countries (Schwartz, Hubschman et al. 2012; Schwartz, Regillo et al. 2014). 22 months after transplantation, the hESC-derived RPE cells showed no signs of rejection or tumourigenicity, which gives a positive outlook on the safe use of pluripotent cells in cell transplantation therapy.

## 1.8 Research proposal

The overall aim of this study was to explore the *in vitro* generation of NK cells from hPSCs and to compare the phenotype of generated cells to UCB CD34<sup>+</sup>-derived and peripheral blood NK cells.

Our hypotheses were that hiPSC-derived NK cells (1) can be generated in numbers comparable to the numbers derived from UCB CD34<sup>+</sup> cells (2) are phenotypically similar to UCB-derived and PBNKs and (3) are functionally active and capable of effectively lysing target cells. HiPSCs could represent an unlimited source of NK cells enabling administration of high cell doses for immunotherapy and supporting the development of a well characterized repository of potentially “off the shelf” cells.

### 1.8.1 Aim 1 - To generate and characterize haematopoietic progenitors from hESCs and hiPSCs.

We hypothesised that haematopoietic progenitors can be efficiently differentiated from various episomal plasmid-derived hiPSC lines. These cells (a) show a similar immunophenotype compared to UCB-derived HSCs, (b) show differentiation potential into the myeloid lineages of the haematopoietic system and (c) can generate NK cells in *in vitro* differentiation protocols.

Objectives:

- To establish and compare protocols for HSC derivation including the co-culture with murine stromal cells, embryoid body formation and 2D culture methods.

- To compare the differentiation efficiencies of hPSC-derived CD34<sup>+</sup> cell populations from different donors.
- To characterize the cell surface marker expression using flow cytometry.
- To assess the myeloid and erythroid differentiation potential using colony forming unit (CFU) assay and compare results in colony numbers to UCB-derived CD34<sup>+</sup> cells.

### **1.8.2 Aim 2 - To establish a robust *in vitro* differentiation protocol for NK cell derivation from hPSC-derived haematopoietic progenitors.**

We hypothesised that hPSC-derived haematopoietic progenitor cells can be efficiently differentiated to NK lymphocytes. These cells (a) can be generated by adopting protocols used for NK cell generation from UCB-derived CD34<sup>+</sup> cells, including EL08.1D2 co-culture and a serum- and feeder free method using GBGM medium which is novel for NK cell derivation from hPSCs, (b) can be generated in numbers comparable to NK cells derived from UCB-derived CD34<sup>+</sup> cells, (c) are phenotypically similar to UCB-derived and peripheral blood NK (PBNK) cells and express a similar receptor repertoire compared to UCB-derived and peripheral blood NK cells and (c) are functionally active and capable of effectively lysing target cells.

Objectives:

- To compare the efficiencies of NK cell derivation methods, including co-culture with EL08.1D2 murine fetal liver cells and a clinically applicable protocol using GBGM medium supplemented with a cytokines cocktail.

- To characterize the cell surface marker expression of hPSC-derived NK cells using flow cytometry.
- To determine the cytolytic efficacy *in vitro* with degranulation experiments using K562 cells as a target.

### **1.8.3 Aim 3 - To explore transcription factor – driven NK cell differentiation by overexpression of E4bp4.**

We hypothesised that the overexpression of human E4bp4 in cord blood and hiPSC-derived CD34<sup>+</sup> cells will increase NK cell numbers with improved cytotoxic effect in NK cell differentiation with GBGM medium. Furthermore, we aimed to investigate if the overexpression of E4bp4 in fibroblasts can activate the NK cell specific gene expression network and directly convert fibroblasts to NK cells.

Objectives:

- To produce functional lentivirus and determine titer.
- To assess the efficiency of lentiviral transduction in UCB- and hiPSC-derived CD34<sup>+</sup> cells and to test if upregulation is stable during differentiation.
- To investigate if cell expansion and phenotype is altered by E4bp4 overexpression using multi-colour flow cytometry.

## Chapter 2 Materials & Methods

### 2.1 Cell culture

All tissue culture was performed in category 2 safety cabinets under sterile conditions. All media were sterile filtered and supplemented with 1mg/ml Penicillin/Streptomycin. Solutions were pre-warmed at room temperature prior to use.

Cell line	Species	Derived from	Received from	Age of patient	Pathological defect in patient	Passage used
<b>OP9</b>	Mouse	Newborn mouse calvaria	ATCC	-	-	22-28
<b>EL08.1D2</b>	Mouse	Murine embryonic liver	Collaboration	-	-	-
<b>HEK 293T</b>	Human	Human embryonic kidney	ATCC	-	-	8-12
<b>Jurkat cells</b>	Human	Peripheral blood	In house	14	T cell leukaemia	-
<b>K562 cells</b>	Human	Peripheral blood	In house	53	CML	-
<b>H1 ESC</b>	Human	Inner cell mass	Collaboration	Pre-implantation	-	63-70

<b>BJ iPSC</b>	Human	Fibroblast s	Derived in our lab (Goh, Caxaria et al. 2013)	Newborn	-	30-50
<b>JOM iPSC</b>	Human	Fibroblast s	Derived in our lab (Goh, Caxaria et al. 2013)	89 years	-	30-50
<b>MMC iPSC</b>	Human	Fibroblast s	Derived in our lab (Goh, Caxaria et al. 2013)	31 years	Rapid onset Parkinsonism Dystonia	30-50
<b>HPS1 iPSC</b>	Human	Fibroblast s	Derived in our lab	4 years	Hermansky Pudlak Syndrome Type 1	30-50
<b>PB3 iPSC</b>	Human	PBMCs	Derived in our lab	Unknown	CEP290	30-50

**Table 2.1 Cell lines.**

### **2.1.1 Human pluripotent stem cells**

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) were maintained in a pluripotent state by passaging them on geltrex (Thermo Fisher Scientific, # A1413302) - coated tissue culture plates in mTESR1 medium (Stem Cell Technologies, #05850) at 37°C/5% CO<sub>2</sub>. Tissue culture plates were coated with geltrex (1% final concentration) for at least one hour before use or alternatively stored at 4°C for up to one week. For passaging, gentle cell dissociation reagent (Stem Cell Technologies, # 07174) was used. Briefly cells were washed once with PBS and 1ml of gentle dissociation medium was added and cells were incubated at room temperature until the edges of the colonies began to curl (up to five minutes).



The enzyme was aspirated and fresh mTESR1 medium was added. Cells were scraped off the plate in small clumps using a 5 ml short glass pipette (Corning). Disaggregated colonies were transferred immediately to geltrex-coated plates. The split ratio was adjusted depending on the growth characteristics of the cells. The cells were split at a 1:2-1:20 ratio. The media was changed every 1 -2 days.

#### **2.1.1.1 Cryopreservation of hPSCs**

Cells were cryopreserved using bambanker freezing medium (Anachem, #95016585). Cells were collected in small clumps as described above and spun down for 5 minutes at 200x g. The cell pellet was resuspended in 100µl mTESR1. 50µl were transferred in each cryovial and 500µl of bambanker freezing medium was added. Cells were immediately transferred to -80°C in a Mr. Frosty to achieve a rate of cooling of -1°C/minute. For long term storage, cells were transferred into a liquid nitrogen tank after a minimum of 24 hours.

#### **2.1.1.2 Thawing hPSCs**

Cryopreserved stem cells were thawed in a 37°C water bath until a small fraction was still frozen. 6ml of pre-warmed mTESR1 medium was added drop-by-drop and cells were spun down for 5 minutes at 200x g. The medium was aspirated and cells were resuspended in fresh mTESR1 and transferred to pre-coated geltrex plate.

#### **2.1.2 OP9 feeder cells**

Murine bone marrow OP9 cells were purchased from ATCC® (Catalog number ATCC® CRL-2749™). The OP9 cell line was established from newborn op/op mouse calvaria. The cells do not produce functional macrophage colony-stimulating factor

(M-CSF) due to an osteopetrotic mutation in the gene encoding M-CSF. The presence of M-CSF had inhibitory effects on the differentiation of stem cells to blood cells other than macrophages.

OP9 cells were maintained on gelatine-coated tissue culture flasks in  $\alpha$ -MEM without ribonucleosides and deoxyribonucleosides made fresh from powder with sodium bicarbonate. This was supplemented with 20% defined FBS HyClone to make the complete growth medium and cells were cultured at 37°C/5% CO<sub>2</sub>. For passaging, medium was discarded and cells were washed twice with PBS. TryPLE (Thermo Fisher Scientific, 12605010) was added and cells were incubated at 37°C for approximately 5 minutes. Cells were resuspended with medium and centrifuged for 5 minutes at 400x g. The supernatant was discarded and cells were transferred into a new flask with fresh medium. Cells were passaged every 4 days using a split ratio of 1:4 and medium was renewed every 2-3 days. For cryopreservation, cells were resuspended in complete growth medium supplemented with 5% DMSO.

### **2.1.3 EL08.1D2 feeder cells**

The murine foetal liver cell line EL08.1D2 was maintained on gelatine-coated tissue culture plates and flasks at 32°C/5% CO<sub>2</sub>. The complete growth medium contained 42.5%  $\alpha$ -MEM, 50% Myelocult (StemCell Technologies M5300), 7.5% FBS, 2mM Glutamax, 10<sup>-6</sup>M Hydrocortisone and 50 $\mu$ M 2-Mercaptoethanol. For passaging, the medium was discarded and cells washed twice with PBS. TryPLE was added and cells were incubated at 37°C for approximately 5 minutes. Cells were resuspended with medium and centrifuged for 5 minutes at 400x g. The supernatant was discarded and cells transferred into a new flask with fresh medium. Cells were passaged every 4 days using a split ratio of 1:4 and medium was renewed every 2-3

days. For cryopreservation cells were resuspended in complete growth medium supplemented with 5% DMSO.

#### **2.1.4 Isolation of human mononuclear cells from peripheral blood and umbilical cord blood**

Umbilical cord blood (UCB) samples were received from the Anthony Nolan institute and peripheral blood samples were obtained from healthy donors in the laboratory. Blood samples were diluted 1:1 with PBS and 35ml was slowly added on top of 15 ml Ficoll - without mixing the two phases. After centrifugation at 400 x g for 30 minutes at RT, without break, the mononuclear cell layer was transferred with a Pasteur pipette into a new tube. The cell suspension was topped up to 50 ml with PBS and centrifuged at 400 x g for 10 minutes at RT. The wash step was repeated twice and mononuclear cell numbers were determined using a haematocytometer. The isolated PBMCs were frozen down in  $\alpha$ MEM/20% FBS/7%DMSO.

#### **2.1.5 Magnetic activated cell sorting (MACS)**

CD34<sup>+</sup> cells were enriched using the CD34 micro beads kit from Miltenyi Biotec (Cat # 130-046-702). Depending on the number of total cells the volumes of reagents and size of separation columns had to be adjusted. The following protocol was used for a total cell number of up to 10<sup>8</sup>. Cells were resuspended in 300 $\mu$ l MACS Buffer containing 0.5% BSA in PBS with 2 mM EDTA (Ethylenediaminetetraacetic acid). For magnetic labelling, the cells were incubated for 30 minutes at 4°C with FcR blocking reagent and CD34 MicroBeads. Cells were washed and resuspended in 500 $\mu$ l buffer. Columns were placed in the magnetic field of a suitable MACS separator and rinsed with buffer. The labelled cell suspension was applied onto the

column and washed 3 times with MACS buffer. Flow-through of unlabelled cells was collected and kept on ice. The column was transferred onto a 15 ml tube, and magnetically labelled cells were flushed out by adding 1ml of buffer and firmly pushing the plunger into the column. Aliquots of CD34<sup>+</sup> cells were further processed for flow cytometry, RNA isolation and CFU assay. The remaining cells were either used fresh for NK cell differentiation or cryopreserved in  $\alpha$ -MEM/20% FBS/7% DMSO.

NK cells were isolated from PBMCs using the NK cell isolation kit from Miltenyi Biotec (Cat # 130-092-657). In brief, up to  $10^7$  PBMCs were resuspended in 40 $\mu$ l MACS buffer and cells were incubated with 10  $\mu$ l of NK cell biotin-antibody cocktail for 5 minutes at 4°C. 30  $\mu$ l of MACS buffer and 20  $\mu$ l of NK cell microbead cocktail were added, mixed well and incubated for an additional 10 minutes at 4°C. The volume was adjusted to 500  $\mu$ l with MACS buffer and the flow through which represented the NK cell fraction was collected after magnetic sorting.

#### **2.1.6 Co-culture with OP9 feeder cells**

OP9 cells were plated on gelatine-coated tissue culture flasks at a density of  $4 \times 10^3$ - $1 \times 10^4$  cells/cm<sup>2</sup> in complete growth medium (see OP9 maintenance). A complete medium change was performed on day 2 and half medium change on day 4 of culture. Cells were overgrown an additional 4 days to form a dense monolayer embedded in extracellular matrix. On day 0 of differentiation  $1.7 \times 10^4$  hPSCs/cm<sup>2</sup> were added as small clumps onto the overgrown OP9 layer in differentiation medium containing  $\alpha$ -MEM without ribonucleosides and deoxyribonucleosides made fresh from powder, supplemented with 20% defined FBS HyClone and 100 $\mu$ M monothioglycerol. A full medium change was performed on day 1 and half medium

changes on day 4 and day 6. Cells were collected on day 9 to 12 by aspirating the medium and incubating with 1mg/ml Collagenase IV solution for 30 minutes at 37°C to digest the collagen-rich matrix. The collagen solution was aspirated and cells were incubated with TryPLE for 15 minutes at 37°C. Cells were collected with complete growth medium and passed through a 0.4µm cell strainer to obtain a single cell suspension. After centrifugation at 300xg for 5 minutes the cell pellet was resuspended in complete growth medium and a cell count was performed using a haematocytometer. An aliquot was kept on ice for Flow Cytometry analysis and remaining cells were prepared for MACS.

#### **2.1.7 Serum- and feeder free differentiation with cytokines**

The differentiation with STEM<sup>TM</sup>diff<sup>TM</sup> APEL<sup>TM</sup> medium from StemCell Technologies is based on the published protocols (Ng, Davis et al. 2008) (Chadwick, Wang et al. 2003). On day 0, approximately 2 days after passaging the pluripotent stem cells, small colonies were present on geltrex-coated plates and medium was changed from mTESR1 to APEL<sup>TM</sup> medium supplemented with 30ng/ml VEGF, 30ng/ml BMP4, 40ng/ml SCF and 50ng/ml Activin A, named APEL A. On day 4 medium was changed to APEL<sup>TM</sup> medium supplemented with 300ng/ml SCF, 300ng/ml Flt3L, 10ng/ml IL3, 10ng/ml IL6, 50ng/ml G-CSF and 25ng/ml BMP4, named APEL B. Media changes were performed on day 7 and day 10 with APEL B and cells were harvested on day 13 by incubating with Accutase for 10mins at 37°C. An aliquot was kept on ice for Flow Cytometry analysis and remaining cells were prepared for MACS.

### **2.1.8 Embryoid body formation**

For embryoid body (EB) formation, pluripotent stem cells were collected in small clumps using cell dissociation reagent and were collected in a 15ml tube. After aggregated cells settled to the bottom of the tube by gravitation, the medium was discarded and cells were transferred to a low-adhesion suspension plate in fresh mTESR1, incubated overnight at 37°C/5% CO<sub>2</sub>. The following day EBs have been formed and medium was changed to APEL™ supplemented with 20ng/ml VEGF, 20ng/ml BMP4 and 40ng/ml SCF to induce haematopoietic mesoderm differentiation. Medium changes were performed on day 4, day 6 and cells were harvested on day 13.

### **2.1.9 Spin embryoid body formation**

In order to generate spin EBs the pluripotent cells were collected as single cells using Accutase. After centrifugation at 200x g for 5 min cells were resuspended at a concentration of 3x10<sup>4</sup> cells/ml in APEL™ medium supplemented with 20ng/ml VEGF, 20ng/ml BMP4 and 40ng/ml SCF. 100µl of this cell suspension were transferred to each of the inner 60 wells of a round-bottomed 96-well plate, giving a final concentration of 3000 pluripotent cells/well. PBS was added to the outer 36 wells of each plate to humidify the EBs during differentiation. Cells were aggregated at the bottom of the wells by centrifugation at 480x g for 4 min which initiated the formation of spin EBs. The plates were placed in an incubator at 37°C/5% CO<sub>2</sub> and the presence of EBs was checked the following day. Medium changes were performed on day 4, day 6 and cells were harvested on day 13.

### **2.1.10 NK cell differentiation**

#### **2.1.10.1 EL08.1D2 co-culture**

CD34<sup>+</sup> cells isolated from cord blood samples or hiPSC-generated CD34<sup>+</sup> cells were co-cultured on a monolayer of irradiated EL08.1D2 murine feeder cells in 96-well plates. 500 CD34<sup>+</sup> cells were plated per well with the following cocktail of cytokines: IL-3 (week one), IL-15, IL-7, SCF in weeks 1-3 and IL-15 only for week 4 and 5. Fresh medium was added weekly and cells were collected at different time points during differentiation for cell counts and flow cytometry analysis.

#### **2.1.10.2 Feeder-free NK cell differentiation with cytokines**

CD34<sup>+</sup> cells isolated from cord blood samples or hiPSC-generated CD34<sup>+</sup> cells were cultured at a density of  $1 \times 10^5$  cells/ml in GBGM (Glycostem basal growth medium) expansion medium (GBGM, 10% Human serum AB, 1x LDC (low-dose cytokine) supplemented with 25 ng/ml SCF, 25 ng/ml Flt3L, 25 ng/ml TPO, 25 ng/ml IL-7 and 20 µg/ml Clivarin). After 10 days of expansion, suspension cells were collected and re-plated in GBGM differentiation medium I (GBGM, 10% Human serum AB, 1x LDC mix, 25 ng/ml SCF, 25 ng/ml Flt3L, 20 ng/ml IL-15, 25 ng/ml IL-7 and 20 µg/ml Clivarin) at a density of at least  $1 \times 10^6$  cells/ml. On day 14 of differentiation, cells were re-plated in GBGM differentiation medium (GBGM 2% Human serum AB, 1x LDC mix, 20 ng/ml SCF, 20 ng/ml IL-15, 20 ng/ml IL-7 and 1000 U/ml IL-2) at a density of at least  $2-3 \times 10^6$  cells/ml. Cell culture medium was refreshed every 2-3 days by replacing 500 µl with fresh medium. 1x LDC mix contained: 10 pg/ml GM-CSF, 250 pg/ml G-CSF and 50 pg/ml IL-6. Cells at different time points during differentiation were collected for cell counts, RNA extraction and flow cytometry analysis.

## 2.2 Phenotypic analysis by flow cytometry

Cells were prepared as a single cell suspension in PBS/0.1% FBS and were labelled with a combination of the following fluorochrome-conjugated monoclonal antibodies (mABs): CD16-APC, CD34-APC, CD43-FITC, CD45-PE, CD56-PE, CD94-PerCPCy5.5, CD133-PE, NKp46-APC and NKG2D-APC all obtained from BD Biosciences and CD117-PerCPCy5.5 from Beckman Coulter. Tra-1-60 and Tra-1-81 antibodies were non-conjugated mouse monoclonal IgM antibodies obtained from Santa Cruz Biotechnology used in combination with an AlexaFluor®488 goat anti-mouse IgG (Thermofischer Scientific). Samples were analysed using a BD FACSVerse™ flow cytometer with BD FACSuite™ and FlowJo software. Control staining with appropriate isotype controls was performed using APC mouse IgG1 κ, FITC mouse IgG1 κ and PE mouse IgG1 κ.

Antibody	Specificity	Clone	Fluorochrome	Company	Catalogue number
<b>TRA-1-85</b>	Mouse anti-human	TRA-1-85	PE	BD Pharmingen	563021
<b>CD3</b>	Mouse anti-human	SK7	APC-Cy7	BD Pharmingen	557832
<b>CD4</b>	Mouse anti-human	SJ25C1	APC-Cy7	BD Pharmingen	557791
<b>CD14</b>	Mouse anti-human	HCD14	PerCP-Cy5.5	BioLegend	325621
<b>CD16</b>	Mouse anti-human	VEP13	VioGreen	Miltenyi Biotec	130-098-095
<b>CD16</b>	Mouse anti-human	3G8	APC-Cy7	BioLegend	302017
<b>CD34</b>	Mouse anti-human	581	APC	BD Pharmingen	555824



<b>CD43</b>	Mouse anti-human	1G10	FITC	BD Pharmingen	555475
<b>CD45</b>	Mouse anti-human	HI30	PE	BD Pharmingen	555483
<b>CD45</b>	Mouse anti-human	5B1	VioBlue	Miltenyi Biotec	130-098-136
<b>CD56</b>	Mouse anti-human	B159	PE-Cy7	BD Pharmingen	557747
<b>CD73</b>	Mouse anti-human	AD2	PE-Cy7	BD Pharmingen	561258
<b>CD90</b>	Mouse anti-human	5E10	PE-Cy7	BD Pharmingen	561558
<b>CD94</b>	Mouse anti-human	DX22	APC	BioLegend	305508
<b>CD133</b>	Mouse anti-human	AC133	PE	Miltenyi Biotec	130-080-801
<b>NKp30 (CD337)</b>	Mouse anti-human	P30-15	APC	BioLegend	325209
<b>NKp46 (CD335)</b>	Mouse anti-human	9E2	VioBright FITC	Miltenyi Biotec	130-104-567
<b>NKp44 (CD336)</b>	Mouse anti-human	P44-8	PE	BioLegend	325107
<b>NKG2A (CD159a)</b>	Mouse anti-human	131411	PE	R&D Systems	FAB1059P
<b>NKG2C (CD159c)</b>	Mouse anti-human	134591	FITC	R&D Systems	FAB138G
<b>NKG2D (CD314)</b>	Mouse anti-human	1D11	PerCP-Cy5.5	BioLegend	320817
<b>CD107a</b>	Mouse anti-human	H4A3	PE	BD Pharmingen	555801

<b>Pan KIR2D</b>	Mouse anti-human	NKVFS1	VioBlue	Miltenyi Biotec	130-099-042
<b>IgG2a, κ</b>	Mouse	G155-178	PE	BD Pharmingen	556653
<b>IgG1, κ</b>	Mouse	MOPC-21	APC-Cy7	BD Pharmingen	557873
<b>IgG1, κ</b>	Mouse	MOPC-21	PE	BioLegend	400111
<b>IgG1, κ</b>	Mouse	MOPC-21	APC	BioLegend	400119
<b>IgG1, κ</b>	Mouse	MOPC-21	FITC	BD Pharmingen	555909
<b>IgG1, κ</b>	Mouse	MOPC-21	PerCP/Cy5.5	BioLegend	400149
<b>IgG1</b>	Mouse	IS5-21F5	VioBlue	Miltenyi Biotec	130-094-670
<b>IgG1</b>	Mouse	IS5-21F5	VioBright FITC	Miltenyi Biotec	130-104-562
<b>IgG2a</b>	Mouse	S43.10	VioBlue	Miltenyi Biotec	130-098-898
<b>IgM</b>	Mouse	IS5-20C4	VioGreen	Miltenyi Biotec	130-100-010
<b>IgG1, κ</b>	Mouse	MOPC-21	PE-Cy7	BD Pharmingen	557872

**Table 2.2 Antibodies used for Flow cytometry.**

### **2.3 Colony forming unit assay**

Colony forming unit (CFU) assays were performed using the StemMACS HSC-CFU media obtained from Miltenyi Biotec. The medium is based on methylcellulose in IMDM (1%), supplemented with FBS (30%) and the following cytokines: stem cell factor (SCF – 50ng/ml), GM-CSF (20ng/ml), G-CSF (20ng/ml), IL-3 (20ng/ml), IL-6 (20ng/ml) and Erythropoietin (Epo- 3U/ml). It supports the clonal progeny of a

single-cell to differentiate and grow in distinct colonies including BFU-E, CFU-E, CFU-GEMM, CFU-G, CFU-M and CFU-GM.  $2 \times 10^3$  CD34<sup>+</sup> cells were added to 2ml StemMACS HSC-CFU media, vortexed vigorously until the cells were well suspended and let tubes sit for 10mins to allow air bubbles to rise. 1ml of the cell suspension was plated in a well of a 12-well plate at a final density of  $1 \times 10^3$  cells/well. The outer wells were filled with PBS to avoid evaporation. Cells were incubated at 37°C/5% CO<sub>2</sub> for 12-14 days.

## 2.4 CD107a degranulation assay

NK cells were incubated with K562 cells in a 1:1 effector: target ratio for 2 hours at 37°C/5% CO<sub>2</sub> in RPMI/10% FCS. Cells were labelled with a CD107a-PE conjugated antibody from BD and expression was determined via flow cytometry.

## 2.5 Molecular Biology

Name	Purpose	Company	Catalogue number
<b>TRizol® reagent</b>	RNA isolation	Life technologies	015596-018
<b>SuperScript®III First-Strand</b>	cDNA synthesis	Life technologies	18080-051
<b>Fast Green SYBR® master mix</b>	qPCR	Thermo Scientific	4385612

**Table 2.3 Reagents used in Molecular Biology**

### 2.5.1 RNA isolation

Cells for RNA isolation were collected as described in passaging of adherent/suspension cells and pelleted by centrifugation. Supernatant was removed

and cell pellets stored at -80°C until RNA extraction. For homogenization, pelleted cells were resuspended in 1ml TRIzol® reagent and incubated at RT for 5 minutes. 0.2ml of chloroform was added per 1ml TRIzol® reagent. Samples were shaken vigorously by hand for 15 seconds. Samples were incubated for 2-3 minutes at RT and centrifuged at 12,000xg for 15 minutes at 4°C. The aqueous phase was transferred into a new tube and RNA precipitated by the addition of 0.5mL of 100% isopropanol. After incubation at RT for 10 minutes, samples were centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed and the gel-like RNA pellet was washed with 75% ethanol, by vortexing briefly and centrifugation at 7,500 x g for 5 minutes at 4°C. After 2-3 washes, the RNA pellet was air-dried for 10-15 minutes at RT and resuspended in 20-50 µl of nuclease-free water. RNA absorbance was measured using nanodrop.

### 2.5.2 cDNA Synthesis

cDNA was synthesised with the “SuperScript® III First-Strand Synthesis System for RT-PCR”. 2µg per RNA sample, 1µl oligo(dT)20 (50 µM) and 1µl dNTP mix (10mM) were mixed in a PCR tube and nuclease-free water was added to bring the final volume to 10µl. The mix was incubated at 65°C for 5 minutes and placed on ice for at least 1 minute. The following cDNA synthesis mix was prepared per reaction:

<b>10x RT buffer</b>	<b>2 µl</b>
<b>25 mM MgCl<sub>2</sub></b>	<b>4 µl</b>
<b>0.1 M DTT</b>	<b>2 µl</b>

<b>RNaseOUT™ (40 U/μl)</b>	1 μl
<b>SuperScript® III RT (200 U/μl)</b>	1 μl

**Table 2.4 cDNA Synthesis Mix.**

10μl of cDNA synthesis mix were added to each RNA/primer sample, mixed, incubated for 50 minutes at 50°C and the reaction terminated at 85°C for 5 minutes. The samples were either kept on ice for qPCR analysis or were stored at -20°C.

### **2.5.3 qRT-PCR**

In each well of a 96-well plate 11μl SYBR® Green master mix plus 0.5μl of forward and 0.5μl of reverse primer were added and mixed with 8μl cDNA sample. The list of primers is listed below in table 2.5. All standards were measured in duplicates and all samples were run in triplicates. Analysis of qPCR data was performed in Microsoft Excel, using the relative standard curve method with GAPDH as an endogenous control by calculating  $2^{-\Delta\Delta CT}$  method. Melting curves were checked after each run to confirm specificity.

Gene	Orientation	Sequence	Product length (bp)
<b>Brachyury (T)</b>	forward	ATGATGGAGGAACCCGGAGA	482
	reverse	ACTGCATCTTTCGGGACCTG	
<b>AFP</b>	forward	TTTGGGACCCGAACTTTCCA	551
	reverse	TTTGCCAATGCTTGGCTCTC	
<b>FoxA2</b>	forward	GGTGTCTGAGGAGTCGGAGA	519
	reverse	AGTTCATGTTGGCGTAGGGG	
<b>Nestin</b>	forward	AGCGTTGGAACAGAGGTTGG	302
	reverse	CCTCTGGGGTCCTAGGGAAT	
<b>Otx2</b>	forward	ATTGCTAGAGCAGCCCTCAC	473
	reverse	CATACCTGCACCCTCGACTC	
<b>Tie2 (TEK)</b>	forward	AGTACGTGGTCCGAGCTAGA	554
	reverse	CACAGCTGGTTCTTCCCTCA	
<b>Lyve1</b>	forward	GACAGTACCTACTCGGTGGC	458
	reverse	AGGCATCGCACGGTAGTTTT	
<b>CD10</b>	forward	GACCTCGTTGACTGGTGGAC	500
	reverse	TCTGTGGTGTTGGCAAGTCT	
<b>PU.1</b>	forward	AGCTTCGCCGAGAACAACCTT	483
	reverse	GGTCATCTTCTTGCGGTTGC	

<b>Klf4</b>	forward	TACCAAGAGCTCATGCCACC	432
	reverse	CTTCCCTCATCGGGAAGACAG	
<b>E4bp4</b>	forward	AAGCTCCGGATCAAAGCCAA	224
	reverse	TGATGCCAGTGCTCCGATTT	
<b>Ets1</b>	forward	AAGAGACCACAGACTTTGAGGGAAG	307
	reverse	CTGCACATTCCATATCTGAGACAC	
<b>Eomes</b>	forward	TCGGCAGGCGCATGTTT	367
	reverse	TTCAAGTCCTCCACGCCATC	
<b>Id2</b>	forward	GCAGCACGTCATCGACTACA	465
	reverse	ACCTTCCAAGTGCAGAAAGGG	
<b>Tbet</b>	forward	GACGGCGGATGTTCCCAT	493
	reverse	TTCTCCCGGAATCCTTTGGC	
<b>Tox</b>	forward	AAGGGCCAAAATCCAAACGC	329
	reverse	TGCATGGCAGTTAGGTGAGG	
<b>CD56</b>	forward	GAACGACGAGGCTGAGTACAT	304
	reverse	GCCAGCCTGTCCTTTCTGTC	
<b>IL-15RB</b>	forward	TGGCACTTCCCAGTTCACAT	307
	reverse	GCGAAGGTTCTCAAAGGGCT	

**Table 2.5 List of primers.**

#### **2.5.4 Protein extraction**

Cellular proteins were extracted and solubilised in RIPA (Radio-Immunoprecipitation Assay) Buffer (Sigma, R 0278). Adherent cells were washed twice with PBS, the appropriate volume of RIPA Buffer (1ml for  $0.5$  to  $5 \times 10^7$  cells) was added and cells incubated on ice for five minutes. Cells were scrapped off and the lysate clarified by centrifugation at  $8,000\times g$  for 10 minutes at  $4^{\circ}\text{C}$  to pellet the cell debris. The supernatant containing soluble protein was carefully transferred into a new tube for analysis or stored at  $-80^{\circ}\text{C}$ . Suspension cells were centrifuged at  $400\times g$  for 5 minutes and the cell pellet washed with PBS twice to remove contaminants. The cell pellet was resuspended in the appropriate volume of RIPA Buffer (1ml per  $0.5$  to  $5 \times 10^7$  cells) and cells were incubated on ice for 5 minutes. The lysates were clarified by centrifugation at  $8,000\times g$  for 10 minutes at  $4^{\circ}\text{C}$  and the supernatant was carefully transferred into a new tube for analysis or stored at  $-80^{\circ}\text{C}$ .

#### **2.5.5 BCA assay**

The total protein in the sample lysates was quantified by BCA protein assays (Pierce TM Protein Assay Kit, Thermo Fisher Scientific, #23225). A set of protein standards was prepared with bovine serum albumin (BSA) according to table 2.6.



<b>Vial</b>	<b>Volume of diluent (RIPA buffer) [μl]</b>	<b>Volume and source of BSA [μl]</b>	<b>Final BSA concentration [μg/ml]</b>
<b>A</b>	0	300 of stock	2000
<b>B</b>	125	375 of stock	1500
<b>C</b>	325	325 of stock	1000
<b>D</b>	175	175 of vial B dilution	750
<b>E</b>	325	325 of vial C dilution	500
<b>F</b>	325	325 of vial E dilution	250
<b>G</b>	325	325 of vial F dilution	125
<b>H</b>	400	100 of vial G dilution	25
<b>I</b>	400	0	0 = blank

**Table 2.6 List of standards used in BCA assay.**

The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). 25 μl of each standard and sample were pipetted as triplicates in a 96-well flat-bottomed microplate. 200 μl of the working reagent were added per well, mixed and incubated at 37°C for 30 minutes. After the plate was cooled down to RT, the absorbance was measured at 562 nm on a plate reader and protein concentration of unknown samples was determined by plotting the standard curve of BSA standards in Microsoft Excel.

### **2.5.6 Western Blotting**

20µl of protein sample were mixed with 10µl loading dye mix (containing 7.5µl LDS sample buffer and 2.5µl DTT reducing agent) and heated to 80°C for 10 minutes. The samples were loaded on a 10% Bis-Tris Protein Gel (Thermo Fisher Scientific, NuPAGE™ Novex™, #NP0301BOX) and run at 200V, 85mA for 40 to 50 minutes, including the protein standard ladder (Novex Sharp pre-stained protein standards, Invitrogen, #LC5800). The gel was removed from the gel tank, placed face down in its plastic housing and the gel casing was cracked open using a spatula. For the transfer, 3 sponges and 3 whatman paper (cut to gel-size) were soaked in transfer buffer and put into the transfer cassette. The gel was placed on top, a piece of Nitrocellulose membrane and 3x whatman paper plus 3x sponges were put on top before placing the cassette into the wet transfer system. The transfer is run at 25V, 125mA and 15W on ice for 1h 30 minutes.

The membrane was stained with Ponceau S for 20 seconds and washed with TBS/tween for 40 minutes (buffer changed 4 times). The membrane was blocked with blocking buffer for 1 hour at RT and incubated with the primary antibody overnight at 4°C. The next day, the membrane was washed with TBS/tween for 40 minutes (buffer changed 4 times) and incubated with the secondary antibody for 1-2 hours at RT. The membrane was washed with TBS/tween for 40 minutes (buffer changed 4 times), incubated with enhanced chemiluminescence (ECL) and the bands were visualised on x-ray film.

#### **2.5.6.1 Buffer**

Running buffer: 50ml 20x stock MOPS + 950 ml DI water, fill the middle part of the tank between two gels and 500µl antioxidant was added

Transfer buffer: 50ml 20x transfer buffer (Bicine, Bis Tris, EDTA) + 200ml methanol + 750 ml DI water and 200 µl antioxidant.

TBS/tween: 50ml 20x stock (20 mM Tris, 127 mM NaCl, HCl until pH 7.4-7.8) + 950 ml DI water + 2% Tween 20

Blocking buffer: 5% skimmed milk powder in TBS/Tween

#### **2.5.6.2 Antibodies**

Primary: E4BP4/NFIL3 (D5K80) Rabbit mAb, #14312 Cell Signaling Technology®, working concentration: 1:1000 in blocking buffer

GAPDH (14C10) Rabbit mAb, #2118 Cell Signaling Technology®, working concentration: 1:1000 in blocking buffer

Secondary: Polyclonal goat anti-rabbit antibody HRP conjugated (Dako, #P0448), working concentration: 1:2000 in blocking buffer

#### **2.5.7 Cloning**

The E4BP4 plasmid was received from a collaborator. For the isolation of high-copy plasmid DNA from *E. coli* cultures, the following Mini-prep kit was used: NucleoSpin® plasmid, Macherey-Nagel, #740588.250. In brief, 2 ml of a saturated *E. coli* LB culture were pelleted, cells resuspended in 250 µl buffer A1 and cells lysed by the addition of 250 µl buffer A2. After 5 minutes of incubation at RT, the tubes were inverted gently – to avoid shearing of genomic DNA – and the lysate clarified by centrifugation at 11000xg for 5 minutes. Plasmid DNA was bound on a NucleoSpin® column by centrifugation at 11000 xg for 1 minute and the silica

membrane was washed with buffer A4. The Plasmid DNA was eluted by adding 50 µl AE buffer and centrifugation at 11000xg for 1 minute at RT. Concentration was determined by Nanodrop analysis. For high-copy plasmid DNA purification of culture volumes of 400 ml, the following Maxiprep kit was used: Midiprep (NucleoBond® Xtra Midi, #740410.100).

### **2.5.8 Restriction digest**

10x NEB buffer

5 µg DNA

5 µl enzyme

Up to 100 µl dH<sub>2</sub>O

1 hour at 37 °C and heat inactivated depending on manufacturing guidelines.

Name of enzyme	Buffer	Restriction site	Catalogue number
<b>Sall</b>	3.1	5' G $\nabla$ TCGAC 3' 3' CAGCT $\wedge$ G 5'	R3138
<b>MluI</b>	3.1	5' A $\nabla$ CGCGT 3' 3' TGCGC $\wedge$ A 5'	R0198
<b>PspXI</b>	CutSmart	5' C $\nabla$ TCGAG 3' 3' CTCGA $\wedge$ G 5'	R0656
<b>BstBI</b>	CutSmart	5' TT $\nabla$ CGAA 3' 3' AAGC $\wedge$ TT 5'	R0519
<b>SmaI</b>	CutSmart	5' GGG $\nabla$ CCC 3' 3' CCC $\wedge$ GGG 5'	R0141
<b>NotI</b>	CutSmart	5' GC $\nabla$ GGCCGC 3' 3' CGCCGG $\wedge$ CG 5'	R3189

**Table 2.7 List of restriction enzymes.**

### **2.5.9 Quick ligation**

Quick ligation buffer	10 $\mu$ l
Cut pCCL.EF1 $\alpha$ .eGFP plasmid	2 $\mu$ l
Cut IRES.E4bp4 plasmid	2 $\mu$ l
H <sub>2</sub> O	up to 20 $\mu$ l
Quick ligase	1 $\mu$ l, Mix, 5 minutes at RT and on ice

### 2.5.10 PCR protocol for cloning

HiFid buffer (5x)	20 µl
dNTPs	2 µl
Phusion	1 µl
Primer OL163	2 µl
Primer OL166	2 µl
DNA (400 ng)	0.59 µl
dH <sub>2</sub> O	72.41 µl

### 2.5.11 Sequencing

Samples were sent for Sanger sequencing by Source Bioscience and the list of sequencing primers can be found below in table 2.8.

Name	Sequence
<b>OL60</b>	3' GAATGCTCGTCAAGAAGACAGGGCCGGTTTC 5'
<b>OL163</b>	5'GGCAACTCGAGTGCCACCATGCAGCTGAGAAAAATGCAGA3'
<b>OL166</b>	5' CCGATCCTCCAGCAACTCCCCGAGAA 3'
<b>MP5860</b>	5' GTTACTGGCCGAAGCCGCTTGGAATAAGG 3'
<b>E4bp4</b>	5' GGCAACTCGAGTGCCACCATGCAGCTGAGAAAAATGCAGA 3'

**Table 2.8 List of sequencing primers.**

## **2.6 Lentivirus work**

### **2.6.1 Transformation**

2µl of ligation were added to 25µl of C2987 competent cells and kept on ice for 20-30 minutes. Cells were heat-shocked at 42°C for 30 seconds and put on ice for 5 minutes. 475µl of super optimal broth (SOC) medium was added and the tube incubated at 37°C for 1 hour, shaking. Cells were plated on LB Agar plates + Kanamycin (final concentration 50µg/ml) and incubated at 37 °C overnight.

### **2.6.2 Transient transfection**

2.5µl PEI were mixed with 47.5µl IMDM, incubated at RT for 5 minutes and 1µg of plasmid DNA was added. After an incubation of 15 minutes at RT, the mix was added drop wise to HEK 293T cells and incubated at 37°C. The transfection was left for 24-48 hours.

### **2.6.3 Preparation of Lentiviral particles**

On day 1 of lentivirus preparation,  $10 \times 10^6$  HEK 293T cells were plated in each 15 cm dish with DMEM/10% FCS. The following day a mix of the 3<sup>rd</sup> generation packaging plasmids was prepared with constitution below in table 2.9.

Plasmid	Concentration [ng/ $\mu$ l]	Per 1 plate [ $\mu$ g]
KGP3R	2046	2,3
RTR2	1952	1,2
VSVG	2327	2,5
pCCL.EF1a.E4BP4.IRES.eGFP	4139	24
DMEM	-	1,6 ml

**Table 2.9 Mix 1 of third generation lentivirus production.**

The mix in tube 2 constituted 60 $\mu$ l of PEI plus 1,5ml DMEM. The mix 1 was sterile filtered into tube 2 with a 0.45  $\mu$ m filter, mixed well and incubated at RT for 20 minutes. 3 ml of the transfection mix was added drop wise per 15 cm dish. On day 3 the medium in each plate was replaced with 15ml fresh DMEM/10% FCS and the supernatant harvested on day 4 and 5 of transfection. The viral supernatant was centrifuged at 400xg for 5 minutes, sterile filtered with a 0.45 $\mu$ m filter and ultra-centrifuged at 25,000 RPM for 1.5 hrs at 4°C. After centrifugation the supernatant was poured off and the pellet gently resuspended in the remaining supernatant. Virus was aliquoted in 20  $\mu$ l aliquots and stored at -80°C.

#### **2.6.4 Determination of lentiviral titer**

On day 1 of virus titration, 8 x 10<sup>4</sup> HEK 293T cells were plated in each well of a 12-well plate with DMEM/10% FCS. On day 2 the medium was replaced with 500 $\mu$ l DMEM/10% FCS per well and a serial dilution of virus was prepared: 1:5, 1:25, 1:125, 1:625 and 1:3125. HEK 293T cells were transduced with 25 $\mu$ l of dilutions 1-



5, leaving one well untransduced as a control. On day 4 the medium was topped up with 500µl DMEM/10% FCS per well and GFP expression was determined by FACS on day 5.

#### **2.6.5 hiPSC transduction**

hiPSCs were passaged in small colonies in 6-well plates. Two days before transduction, an approximate cell number was determined by single cell dissociation of one well using Accutase. Lentiviral particles were added drop wise onto the cells in a small amount of medium (1ml / well of a 6-well plate). The cells were incubated at 37°C / 5% CO<sub>2</sub> and 1 ml of fresh mTESR1 was added the following day. On day 3 the transduction efficiency was determined by GFP expression analysis using flow cytometry and a full medium change was performed. Cells were either used for differentiation experiments directly or were expanded in their pluripotent state using mTESR1.

#### **2.6.6 Statistical analysis**

Statistical comparisons were performed with GraphPad Prism software using one-way analysis of variance (one-way ANOVA) followed by non-parametric test. Results are presented as Mean±SD and p values of <0.05 (\*) were considered statistically significant.

## **Chapter 3 Generation and characterisation of haematopoietic progenitors from hPSCs**

### **3.1 Introduction**

The *in vitro* derivation of haematopoietic progenitors and mature blood cells from hiPSCs holds great promise for basic research and clinical applications. It provides an *in vitro* platform to study the generation of blood progenitors and gain better understanding of related disease development. Furthermore, the potential use of hPSCs as an alternative cell-source for stem cell transplantations and immunotherapies is an alluring goal in regenerative medicine. In recent years, great progress has been made to identify the underlying mechanisms of haematopoietic lineage specification and various protocols for *in vitro* derivation have been developed.

#### **3.1.1 Haematopoietic stem cell specification *in vivo***

The key to successful modelling of haematopoietic development *in vitro* is a detailed understanding of what happens *in vivo*. Studies of mouse ESC differentiation and embryogenesis revealed key pathways of the early development of haematopoietic progenitors *in vivo*. Gastrulation is the first important step in development of blood precursors. This step is defined by the formation of the primitive streak (PS) in which epiblast cells migrate into and give rise to the mesoderm and definitive endoderm (Kinder, Tsang et al. 1999). During embryogenesis, endothelial and haematopoietic progenitor cells develop in parallel from a common precursor called haemangioblast, which is defined as aggregates of yolk sac mesenchyme (Choi, Kennedy et al. 1998). A subset of cells with haemato-endothelial potential (KDR<sup>+</sup>)

migrates into the yolk sac, where the first blood progenitors are formed (Yamaguchi, Dumont et al. 1993). It has been shown that BMP4, Nodal, WNT3 and FGF2 are the most important factors for mesoderm induction (Conlon, Lyons et al. 1994; Winnier, Blessing et al. 1995; Liu, Wakamiya et al. 1999; Saxton and Pawson 1999). In the mouse embryo, the first haematopoietic stem cells (HSCs), which are capable of reconstituting the entire haematopoietic system of adult mice are identified within the aorta-gonado-mesonephros (AGM) region, umbilical arteries and placenta by E10.5-11 and it is estimated that this region contains only around 1 HSC at E11.5 (Medvinsky and Dzierzak 1996; Kumaravelu, Hook et al. 2002; Ottersbach and Dzierzak 2005). Studies in mice revealed, that within the AGM, haematopoietic cells were found to bud off from a unique population of endothelial cells on the ventral wall of the dorsal aorta, defined as the hemogenic endothelium (HE) through a transition of endothelial to haematopoietic cells (Choi, Kennedy et al. 1998; Chen, Yokomizo et al. 2009; Boisset, van Cappellen et al. 2010). Subsequent studies showed that other embryonic and extraembryonic sites possess hemogenic potential such as the placenta or head vasculature (Gekas, Dieterlen-Lievre et al. 2005; Li, Lan et al. 2012). The expansion of HSCs occurs in the fetal liver and cells later migrate to the bone marrow, which represents the site of adult haematopoiesis (Cumano and Godin 2007).

### **3.1.2 *In vitro* HSC differentiation from pluripotent stem cells**

To fully understand the early steps of human haematopoiesis and the dissimilarity to the mouse model, *in vitro* modelling with hPSCs serves as a powerful system. Three major approaches for the *in vitro* derivation of human HSCs have been described in the literature: (1) the co-culture with mouse stromal cell lines, (2) embryoid body (EB) formation and (3) 2D culture protocols.

Different murine stromal cell lines have been compared for their efficiency to induce haematopoietic differentiation, including S17, M210-B4 and MS-5 cell lines (Vodyanik, Bork et al. 2005) (Ni, Knorr et al. 2011) (Woll, Martin et al. 2005). The co-culture on OP9 stromal cells appeared most efficient for HSC derivation and differentiation into myeloid, erythroid and lymphoid lineages (Vodyanik, Bork et al. 2005; Choi, Vodyanik et al. 2011). The OP9 cell line was established from newborn op/op mouse calvaria. The cells do not produce functional macrophage colony-stimulating factor (M-CSF) due to an osteopetrotic mutation in the gene encoding M-CSF. The presence of M-CSF had inhibitory effects on the differentiation of PSCs to blood progenitors other than macrophages (Nakano 1996). Studies suggest that CD34<sup>+</sup>CD43<sup>+</sup> and CD34<sup>+</sup>CD45<sup>+</sup> cell populations contain more haematopoietic progenitors compared to CD34<sup>+</sup>CD43<sup>-</sup> and CD34<sup>+</sup>CD45<sup>-</sup> (Woll, Martin et al. 2005) (Knorr, Ni et al. 2013).

EB formation mimics the differentiation within a compact structure like the embryo proper in *in vitro* culture conditions. PSCs differentiate randomly into multiple cell types and differentiation can be forced into a specific lineage by adding cytokines and small molecules. Haematopoietic development has been significantly enhanced by the addition of bone morphogenetic protein-4 (BMP-4) and the haematopoietic cytokines stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), interleukin-3 (IL-3), interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) (Chadwick, Wang et al. 2003). In order to perform a uniform differentiation of PSCs, a variation of the EB protocol has been developed using an animal product-free medium, denoted APEL (Albumin Polyvinylalcohol Essential Lipids) or the more economical variant, BPEL (Bovine Serum Albumin Polyvinylalcohol Essential Lipids) in combination with the spin EB methodology (Ng, Davis et al. 2008; Knorr, Ni et al. 2013). A variation of the EB protocol uses BPEL medium in combination with SCF, BMP-4 and VEGF only (Knorr, Ni et al. 2013).

Protocols for deriving haematopoietic progenitors in 2D cultures have been explored using media and cytokines similar to the EB formation protocols. These protocols are serum- and feeder free and cells are grown as a monolayer culture with regular media changes supplemented with cytokines (Chicha, Feki et al. 2011; Niwa, Heike et al. 2011; Salvagiotto, Burton et al. 2011). A haematopoietic differentiation protocol using STEMdiff APEL medium from Stemcell Technologies has been published based on the protocols of EB formation (Chadwick, Wang et al. 2003; Ng, Davis et al. 2008).

### **3.1.3 Challenges and obstacles of hiPSC-derived HSC derivation**

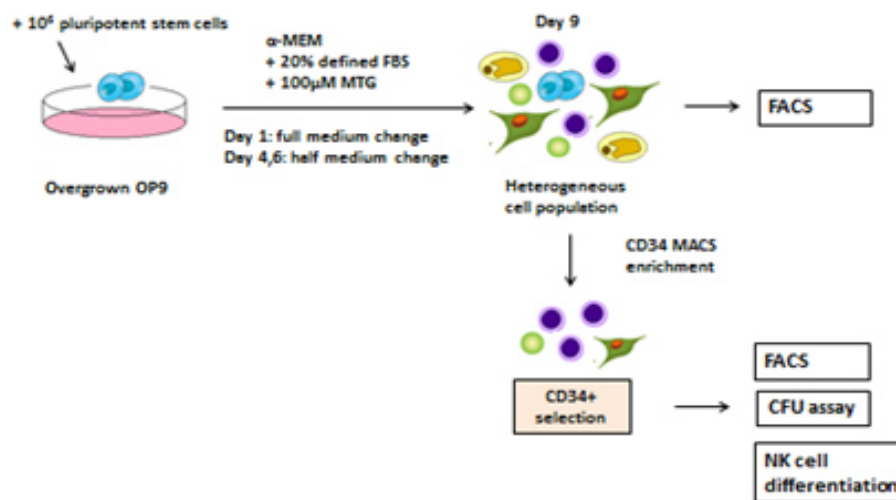
Although there has been a lot of progress in the research field in recent years, the differentiation efficiencies remain low and generation of hiPSC-derived HSCs with engraftment and long-term reconstitution potential remain a big challenge in the field (Wang, Menendez et al. 2005; Narayan, Chase et al. 2006; Tian, Woll et al. 2006; Ledran, Krassowska et al. 2008; Tian, Hexum et al. 2009; Risueno, Sachlos et al. 2012). Data suggest that the current protocols are limited and new approaches for overcoming these obstacles are required. Recent studies investigate the effect of over-expression of haematopoietic key transcription factors on reconstitution efficiency of hiPSC-derived HSCs. Ran et al. showed that the over-expression of RUNX1a enhances haematopoietic lineage commitment in hiPSC-derived differentiation cultures and improves multilineage reconstitution *in vivo* (Ran, Shia et al. 2013). Direct TF-driven approaches demonstrate that the over-expression of key transcriptional regulators can induce haematopoietic lineages from hiPSCs. Elcheva et al. (Elcheva, Brok-Volchanskaya et al. 2014) reported in their study that the haemato-endothelial program can be induced in hiPSCs by the expression of a set of transcriptional regulators: ETV2 and GATA2 for the pan-myeloid and GATA2 and

TAL1 for the erythro-megakaryocytic lineage. In another study, the direct-programming which circumvents the transition through a pluripotent intermediate of endothelial to haematopoietic cells is reported (Sandler, Lis et al. 2014). By over-expressing the transcription factors FOSB, GFI1, RUNX1 and SPI1 in endothelial cells, this group demonstrated the derivation of multipotent blood progenitor cells which successfully engrafted into immune-deficient mice; producing both myeloid and lymphoid cells. A recent study describes a combined approach of morphogen-driven and transcription factor-mediated differentiation of hPSCs that results in HSCs with engraftment potential (Sugimura, Jha et al. 2017). In this study, cells with engraftment, self-renewal and multi-lineage differentiation potential have been generated from hPSCs. A set of seven transcription factors – RUNX1, ERG, LCOR, HOXA5, HOXA9, HOXA10 and SPI1 - has been identified by *in vivo* screening. This set of TFs facilitates the differentiation of *in vitro*-hPSC-derived haemogenic endothelial cells to HSCs that engraft myeloid, B and T cells in primary and secondary mouse recipients. RNA sequencing analysis of engrafted cells revealed a strong correlation to haemogenic endothelium, engrafted cord blood HSCs and fresh cord blood CD34<sup>+</sup> cells suggesting the activation of transcription factor networks that reflect the capacity for engraftment and reconstitution in transplant recipients (Sugimura, Jha et al. 2017).

## **3.2 Results**

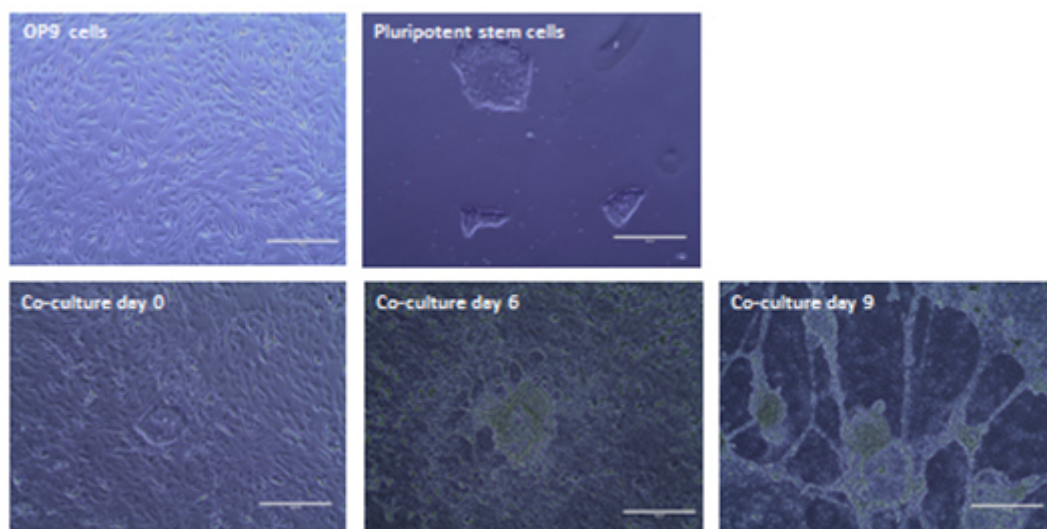
### **3.2.1 Haematopoietic stem cell progenitors derived with co-culture on murine OP9 stromal cells**

All hiPSC lines used in this study have been derived by episomal plasmid reprogramming in our lab and have been extensively characterised (Goh, Caxaria et al. 2013), see table 2.1. In order to establish a robust and reproducible protocol for the differentiation of hPSCs into HSCs, we adapted the protocol from Choi et al. and Vodyanik et al. and their differentiation studies on hPSCs to HSCs and myeloid progenitors (Vodyanik, Bork et al. 2005; Choi, Vodyanik et al. 2011). In this study, we aimed to compare the differentiation potential of the hESC line H1, hiPSC line derived from the newborn foreskin fibroblast cell line BJ and four patient-derived hiPSC lines: JOM-iPSC, MMC-iPSC and HPS1-iPSC were derived from patient fibroblasts and PB3-iPS cells from peripheral blood mononuclear cells (PBMCs) (Goh, Caxaria et al. 2013). In brief, hPSCs were added in small clumps on an over-confluent OP9 stromal cell layer and cells were cultured over a period of 9 days (Figure 3.1). Typical morphological changes could be observed within all hPSC cultures (Figure 3.2). Stem cell colonies started to expand on the stromal cell layer by day 1, the centre and edges began to differentiate within the first four to six days of differentiation. This developmental stage is believed to be the equivalent to the haemangioblast, a mesenchymal structure described in early development (Choi, Kennedy et al. 1998). After about eight days of co-culture, distinct radial sac-like structures were formed.



**Figure 3.1 Experimental layout of OP9 co-culture differentiation protocol.**

hiPSCs are seeded in small colonies on an overgrown OP9 cell layer. On day 9, a heterogeneous cell population is collected for characterisation, CD34<sup>+</sup> cell enrichment and further differentiation.

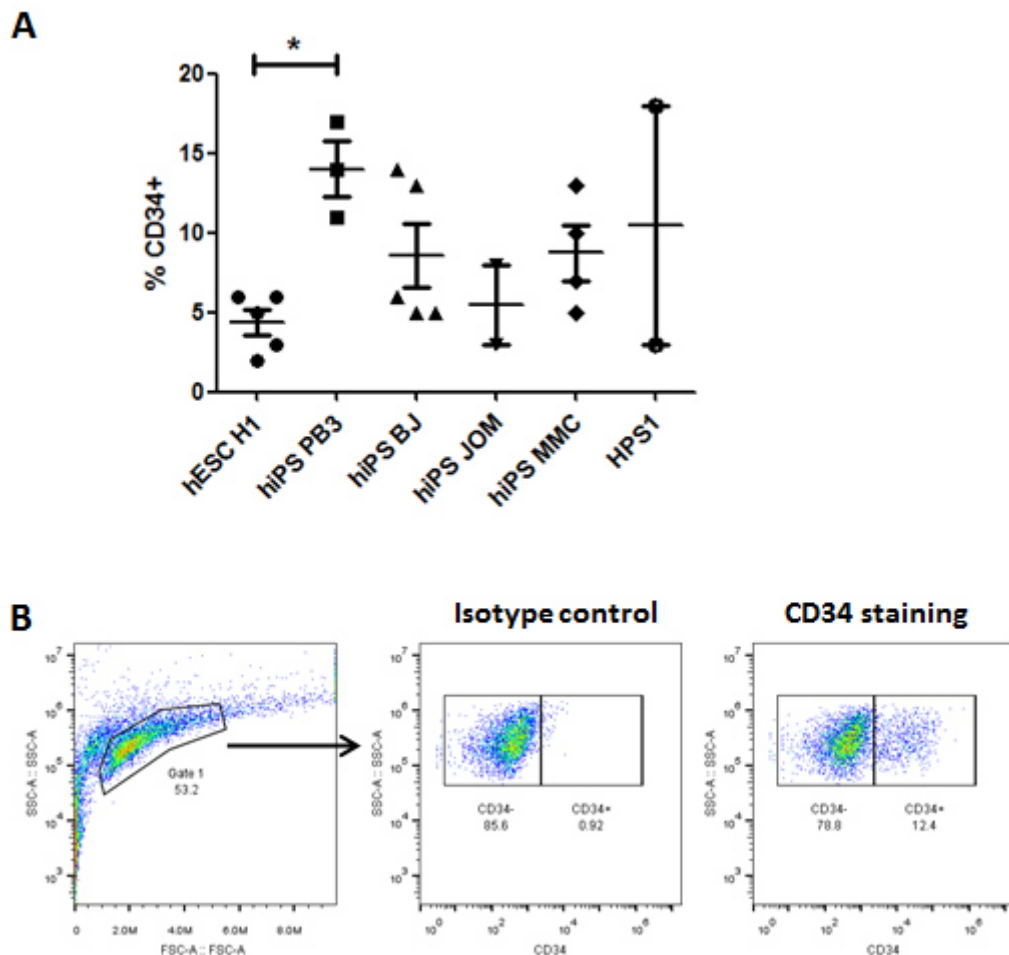


**Figure 3.2 Morphological changes in OP9 co-culture at days 0, 6 and 9.**



### 3.2.2 CD34<sup>+</sup> cells can be generated from hPSCs

All cell lines tested were capable of generating haematopoietic progenitor cells assessed by the expression of CD34 (Figure 3.3). The efficiency of  $4,4 \pm 1,8\%$  CD34<sup>+</sup> of the control hESC line H1 was lower compared to published data of  $12,8 \pm 4,7\%$ , achieved using the same protocol (Vodyanik, Bork et al. 2005). The differences in efficiency may be due to the use of different starting cell lines and lab environment.



**Figure 3.3 CD34 efficiencies with OP9 co-culture.** (A) CD34<sup>+</sup> efficiencies of hESC line H1 ( $4,4 \pm 1,8\%$ ,  $n=5$ ), and five different hiPSC lines: PB3 ( $14 \pm 3\%$ ,  $n=3$ ), BJ ( $8,6 \pm 4,5\%$ ,  $n=5$ ), JOM ( $5,5 \pm 3,5\%$ ,  $n=2$ ), MMC ( $8,75 \pm 3,5\%$ ,  $n=4$ ) and HPS1

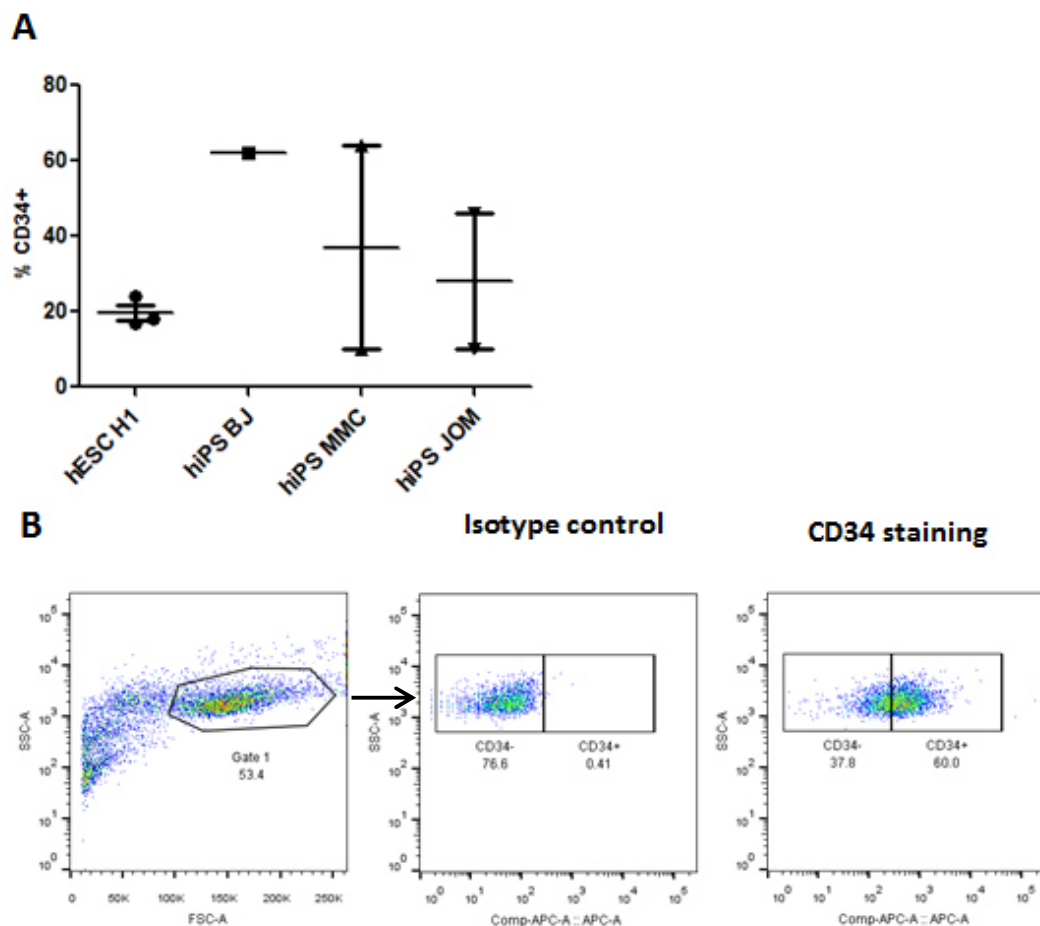
( $10,5 \pm 10,6\%$ ,  $n=2$ ) are shown. (B) The percentage of CD34<sup>+</sup> cells is based on flow cytometry analysis. The gating strategy is shown on the example of one experiment of BJ hiPSCs. Results are presented as Mean $\pm$ SD,  $p<0.05(*)$ . Statistical testing was performed with one-way analysis of variance (one-way ANOVA) followed by non-parametric test.

Although the forward differentiation efficiencies varied between experiments, in general we observed higher HSC differentiation with hiPSCs when compared to hESCs, although this difference is not statistically significant. The fibroblast-derived hiPSC lines yielded comparable numbers of CD34<sup>+</sup> cells with  $8,6 \pm 4,5\%$  for BJ hiPSCs  $5,5 \pm 3,5\%$  for JOM hiPSCs,  $8,75 \pm 3,5\%$  for MMC hiPSCs and  $10,5 \pm 10,6\%$  for HPS1 hiPSCs. In contrast, significantly ( $p=0,0012$ ) higher numbers of CD34<sup>+</sup> cells were obtained from the PBMC-derived hiPSC line PB3 with  $14 \pm 3\%$  of CD34<sup>+</sup> cells.

### **3.2.3 Feeder- and serum-free HSC differentiation with APEL medium**

To explore options for clinically applicable feeder- and serum free HSC derivation we opted for a protocol based on (Ng, Davis et al. 2008) (Chadwick, Wang et al. 2003) and their studies on EB formation with addition of haematopoietic cytokines. The basal medium was obtained from StemCell Technologies named APEL and the components for a more economical variant called BPEL is published in Ng et al. (Ng, Davis et al. 2008). Stem cell colonies remain in 2D culture with APEL medium and cytokines for 13 days. The control hES cell line H1 showed consistent differentiation efficiency of  $20 \pm 3\%$  of the resulting population expressing CD34. However we observed high variability in CD34 expression in 3 hiPS cell lines used

to derive CD34<sup>+</sup> cells with this protocol (Fig. 3.4). Notably, the CD34<sup>+</sup> expressing cell population generated from hESC H1 is greater compared to OP9 co-culture derived cells, however, and CD34 expression appears as a shift rather than a distinct population.

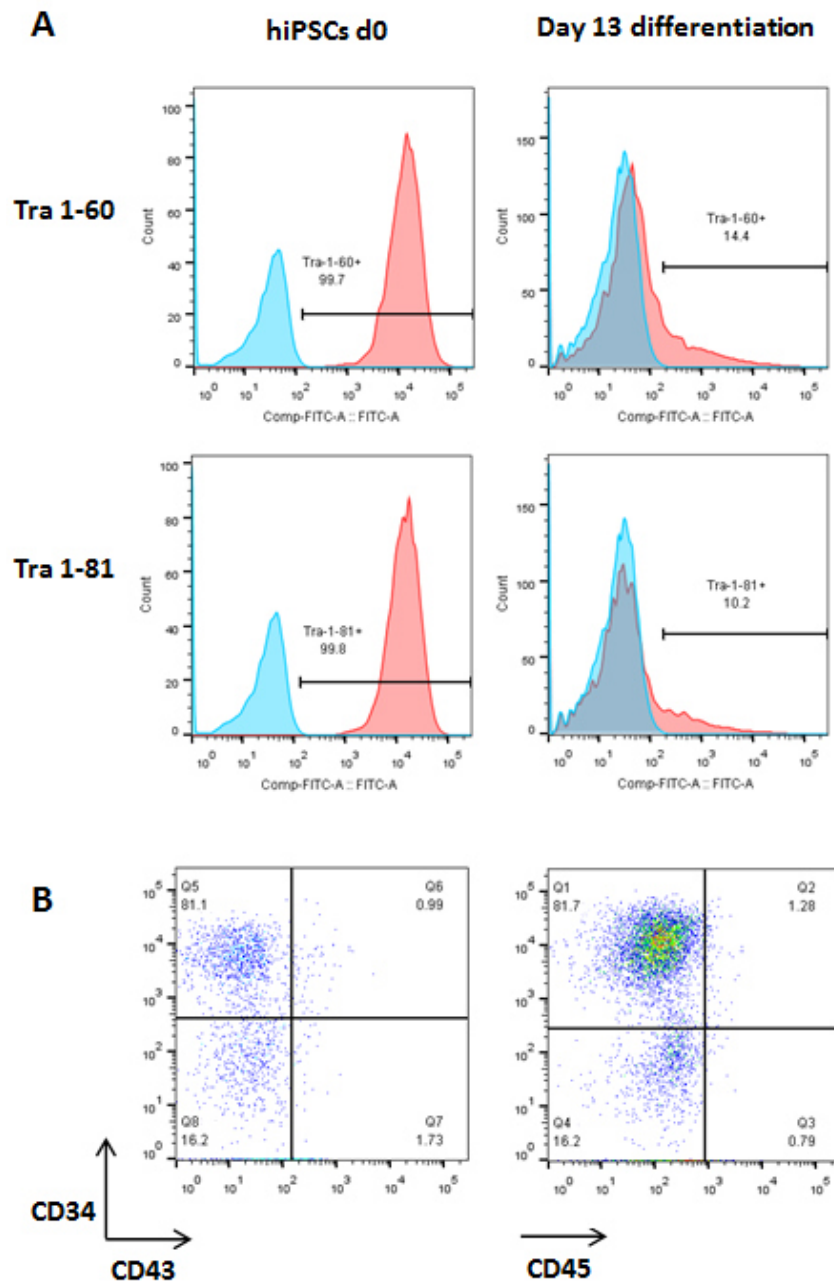


**Figure 3.4 CD34 efficiencies with serum- and feeder free differentiation.**

(A) CD34 efficiencies before MACS enrichment in serum- and feeder free differentiation conditions based on flow cytometry. High variability in two hiPS cell lines (MMC and JOM) could be observed. (B) The gating strategy is shown on the

example of one experiment of BJ hiPSC differentiation. Results are presented as Mean $\pm$ SD. HESC H1 n=3, hiPSC BJ n=1, hiPSC MMC n=2 and hiPSC JOM n=2.

To assess if any cells remained in a pluripotent state, we stained the differentiated cells before MACS enrichment with the pluripotency markers Tra-1-60 and Tra-1-81. On day 13 of differentiation,  $14 \pm 0,6\%$  (n=3) expressed Tra-1-60 and  $10 \pm 1,2\%$  Tra-1-81 (Figure 3.5 A). The hESC H1-derived CD34 expressing cell population could be enriched by MACS to 81% (n=1) purity. However, we did not observe co-expression of the haematopoietic markers CD43 and CD45 (Figure 3.5 B). We therefore surmised that the CD34<sup>+</sup> cells generated with this protocol most likely generated CD34<sup>+</sup> cells that were programmed towards either the endothelial or mesenchymal lineage. In support of this we did not observe any colonies when these feeder and serum-free CD34<sup>+</sup> cells were cultured in methyl cellulose. We conclude that although higher efficiencies can be achieved with the serum- and feeder free approach, the derived cells did not acquire haematopoietic differentiation potential in our preliminary results.



**Figure 3.5 Pluripotency and haematopoietic marker expression.** (A) The expression of pluripotency markers Tra-1-60 and Tra-1-81 decreased significantly during differentiation. Tra-1-60 and Tra-1-81 were still expressed in  $14 \pm 0,6\%$  and  $10 \pm 1,2\%$  of CD34 MACS enriched cells. Blue and red histograms represent the isotype control and specific staining, respectively. (B) No co-expression of CD43 and CD45 could be observed in the serum- and feeder free differentiation approach. Histograms and blots are representative of 3 experiments.

### 3.2.4 Embryoid body formation

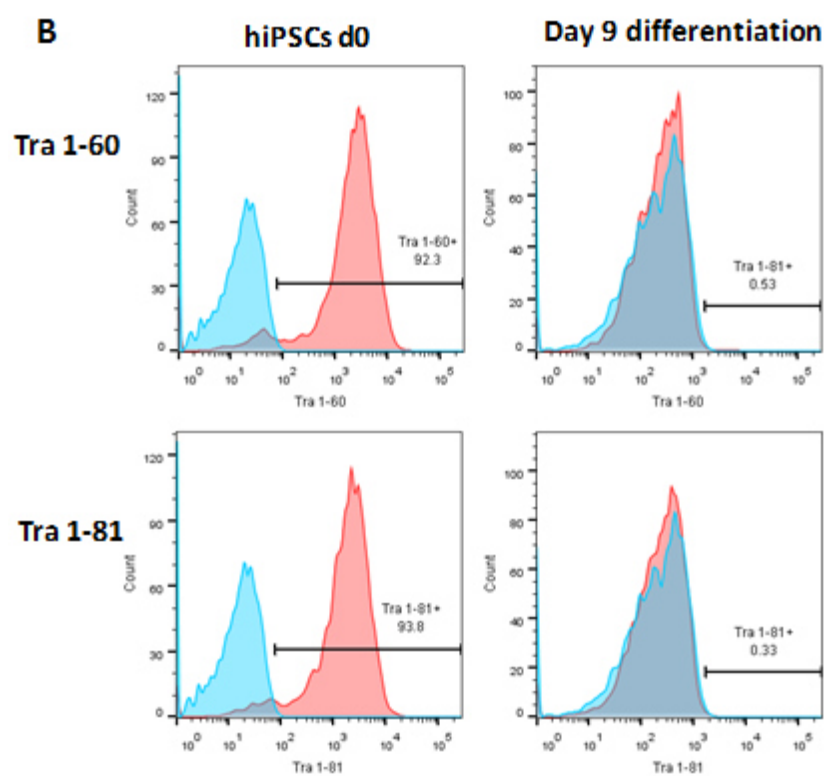
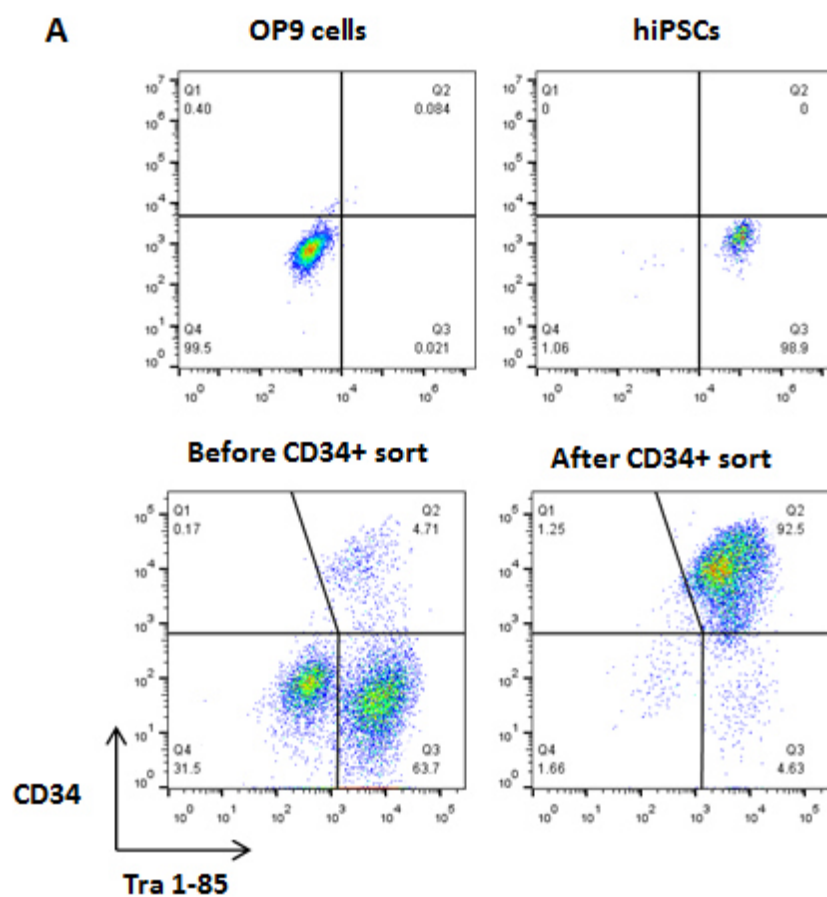
Possibly, the differentiation with EB formation provides an environment closer to the *in vivo* development than 2D cultures can provide. EBs could successfully be derived from hESCs and hiPSCs and were cultured under mesoderm/haematopoietic inducing conditions (see chapter 3.2.3). EBs are very compact, three-dimensional aggregates with complex cell-adhesions. In our experiments we could not successfully dissociate the EBs into a live single cell suspension. Due to this problem we could not analyse the marker expression via FACS and could not enrich the haematopoietic precursors with magnetic beads. Several enzymes for dissociation were tested, including Accutase, trypsin/EDTA, tryPLE and gentle dissociation reagent. To improve the dissociation step in this protocol an “Embryoid body dissociation kit” could be tested available from Miltenyi Biotec.

Based on our differentiation studies of hiPSCs to HSCs we decided to conduct further characterisation and experiments with OP9 co-culture derived CD34<sup>+</sup> cells as this method was most robust and showed consistent differentiation efficiencies.

### 3.2.5 CD34<sup>+</sup> cell enrichment and immunophenotype characterisation

For further analysis of hiPSC-derived CD34<sup>+</sup> cells, a more homogenous cell population was obtained by cell enrichment with CD34 magnetic beads using magnetic activated cell sorting (MACS) technology. An absolute number of  $3,8 \pm 1,7 \times 10^5$  (n=12) CD34<sup>+</sup> cells could be isolated from  $3 \times 10^6$  PSCs, with a cell purity of  $90 \pm 2.6\%$  CD34<sup>+</sup> cells. To distinguish murine and human cells within the co-culture, cells were co-stained with the pan-human cell marker Tra-1-85. As expected, OP9

cells stained negative and hiPSCs positive for Tra-1-85 in the starting cell populations. After nine days of co-culture, all CD34<sup>+</sup> cells co-expressed Tra-1-85 which confirmed the human origin of these cells (Figure 3.6).



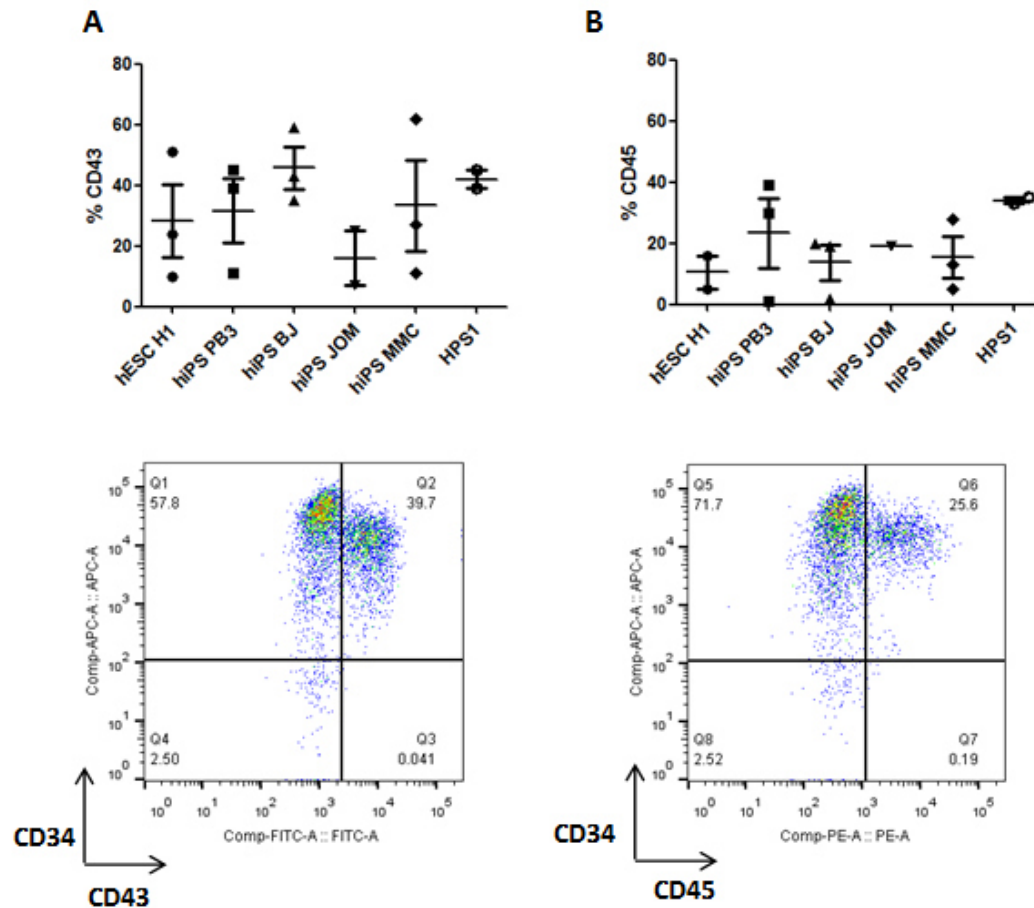


**Figure 3.6 Pan human and pluripotency marker expression.** (A) The expression level of CD34 and the pan-human marker Tra-1-85 in OP9 cells, hiPSCs, after nine days of co-culture and after CD34<sup>+</sup> cell enrichment. Gates were set with the appropriate isotype controls. (B) The expression of pluripotency markers Tra-1-60 and Tra-1-81 decreased significantly during differentiation and CD34<sup>+</sup> sorted cells did not show any expression of pluripotency markers. Blue and red histograms represent the isotype control and specific staining, respectively.

Although CD34 is a commonly used marker for HSCs, it is also expressed by endothelial and mesenchymal cells. To further assess the percentage of cells with haematopoietic differentiation potential, we assessed the co-expression of the cell surface markers CD43, CD45, CD117 and CD133. Previous studies on hESC differentiation with OP9 stromal cells have demonstrated that the early haematopoietic progenitors could be identified by the expression of CD43 which distinguishes haematopoietic CD34<sup>+</sup> cells from endothelial (CD34<sup>+</sup> CD31<sup>+</sup> CD43<sup>-</sup> KDR<sup>+</sup>) and mesenchymal (CD34<sup>+</sup> CD31<sup>-</sup> CD43<sup>-</sup>) cells (Vodyanik, Thomson et al. 2006).

We identified the percentage of CD43 co-expression in CD34-enriched cell populations and observed a wide variation between experiments with numbers ranging from 5-60% (Fig. 3.7A). The acquisition of CD45 occurs later in development and CD34<sup>+</sup>/CD45<sup>+</sup> cells were described as a population highly enriched in myeloid progenitor cells. In our experiments we observed varying expression of CD45 within the enriched CD34<sup>+</sup> cell population similar to CD43 expression (Fig. 3.7B). CD45 is co-expressed on all CD34<sup>+</sup> cells of UCB-derived HSCs (data not shown) and we observed expression of this marker within the CD34<sup>dim</sup> population of hESC-derived cells. Altogether these data suggest that only a

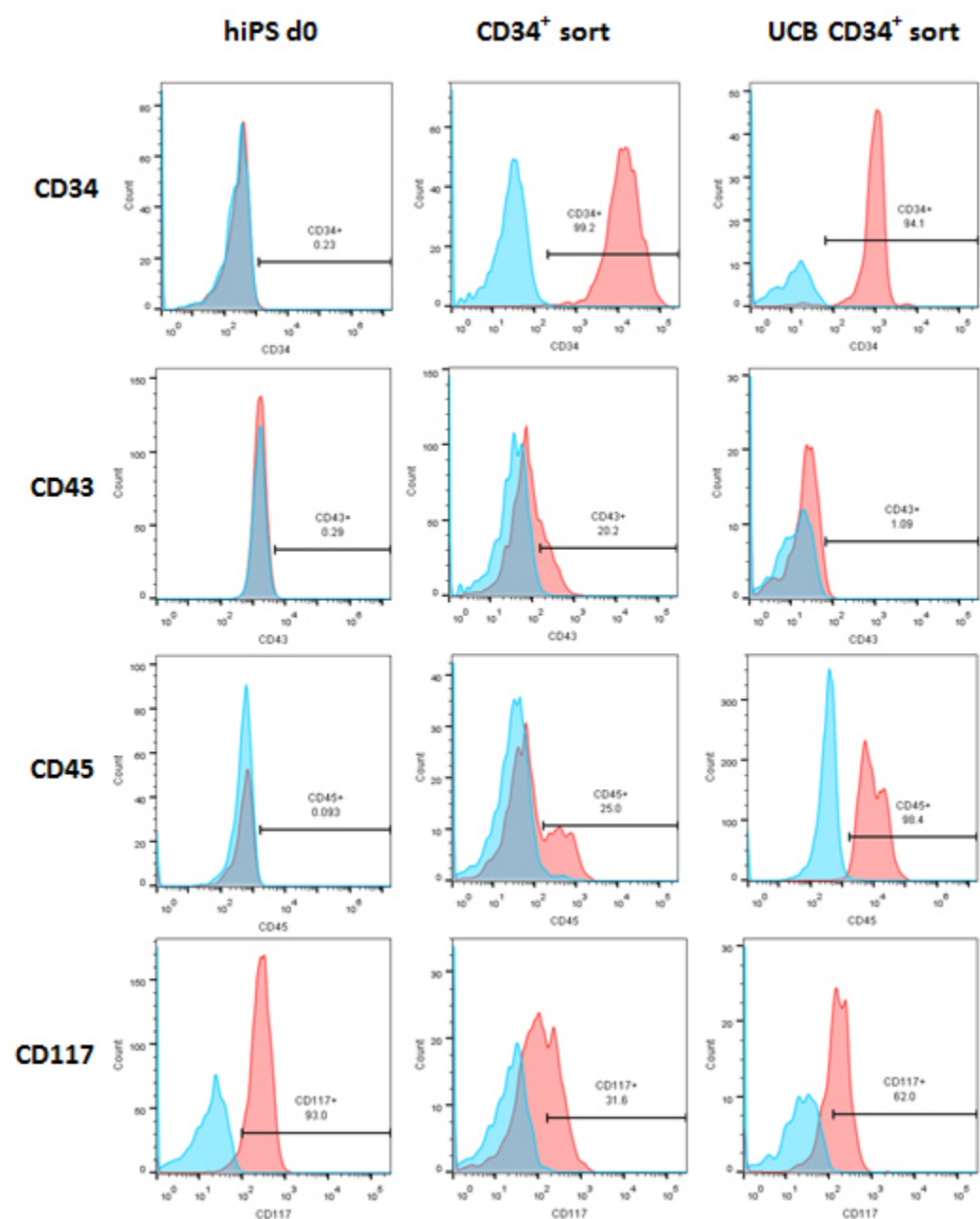
small fraction of isolated hPSC-derived CD34<sup>+</sup> cells are committed to the haematopoietic lineage.

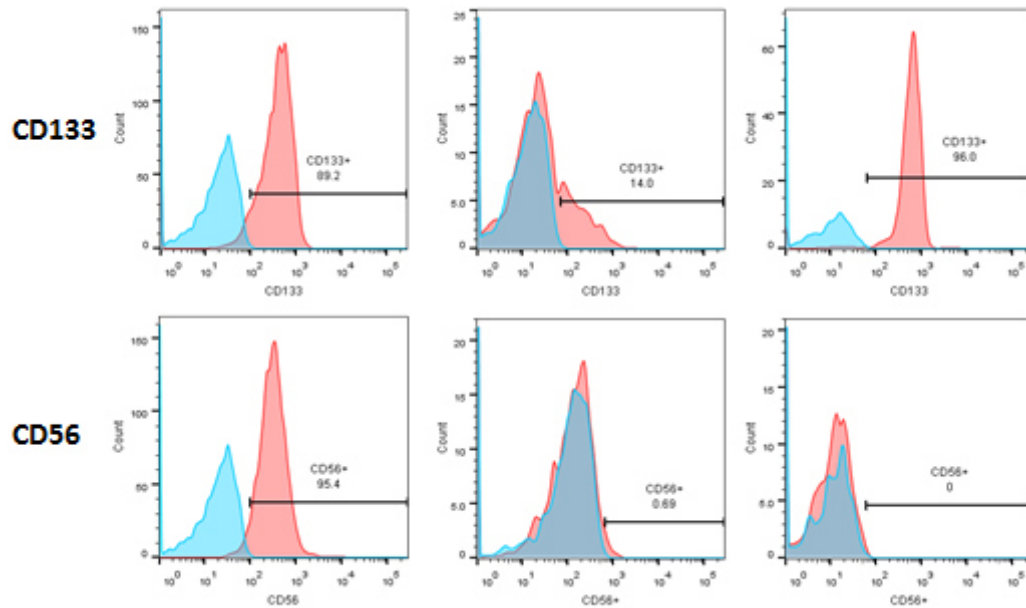


**Figure 3.7 CD43 and CD45 co-expression.** Percentages of co-expression of haematopoietic markers CD43 (A) and CD45 (B) in one hESC and five hiPSC lines are shown. FACS plot examples of a BJ hiPSC line are shown. The data represent the percentage of CD43, CD45 marker expression in 100% CD34<sup>+</sup> cell population. Gates were set using the appropriate isotype controls. Results are presented as Mean ± SD.

### **3.2.6 HiPSC-derived CD34-expressing cells compared to UCB-derived CD34<sup>+</sup> cells**

To further characterise the CD34<sup>+</sup> - enriched cell population derived from hiPSCs, we compared cell surface marker expression with hiPSCs and CD34<sup>+</sup> cells isolated from umbilical cord blood. The haematopoietic markers CD34, CD43 and CD45 were not expressed in the pluripotent cells at the beginning of differentiation, with  $0,2 \pm 0\%$  CD34,  $0,3 \pm 0\%$  CD43 and  $0,1 \pm 0\%$  CD45 (n=2) (Figure 3.8). All three markers were upregulated within 9 days of OP9 co-culture. After MACS enrichment, purities of  $98,6 \pm 0,6\%$  and  $95,7 \pm 1,6\%$  CD34 expressing cells can be achieved in hiPSC- and UCB-derived cells, respectively. CD43 expression could not be detected in UCB-derived CD34<sup>+</sup> cells ( $1,2 \pm 0,1\%$ ) but is slightly upregulated in hiPSC-derived cells with  $19,4 \pm 0,8\%$ . In contrast, CD45 is expressed in  $97,6 \pm 0,8\%$  of UCB-derived CD34<sup>+</sup> cells and to a smaller amount in hiPSC-derived cells with  $24,4 \pm 0,6\%$ . The stem cell markers CD117 and CD133 could be detected in hiPSCs as well as in the CD34<sup>+</sup>-enriched cell populations. CD117 was less expressed in hiPSC-derived cells with  $31,3 \pm 0,3\%$  compared to  $66 \pm 4\%$  observed in UCB-derived samples. Downregulation of CD133 could also be observed in hiPSC-derived cells with  $16,8 \pm 2,8\%$  compared to  $95,4 \pm 0,6\%$  in UCB-derived cells. We detected CD56 expression in pluripotent stem cells with  $96,1 \pm 0,7\%$ , which was downregulated to  $0,8 \pm 0,1\%$  in hiPSC-derived CD34<sup>+</sup> cells which is comparable to expression levels in UCB-derived CD34<sup>+</sup> cells ( $0,3 \pm 0,3\%$ ).





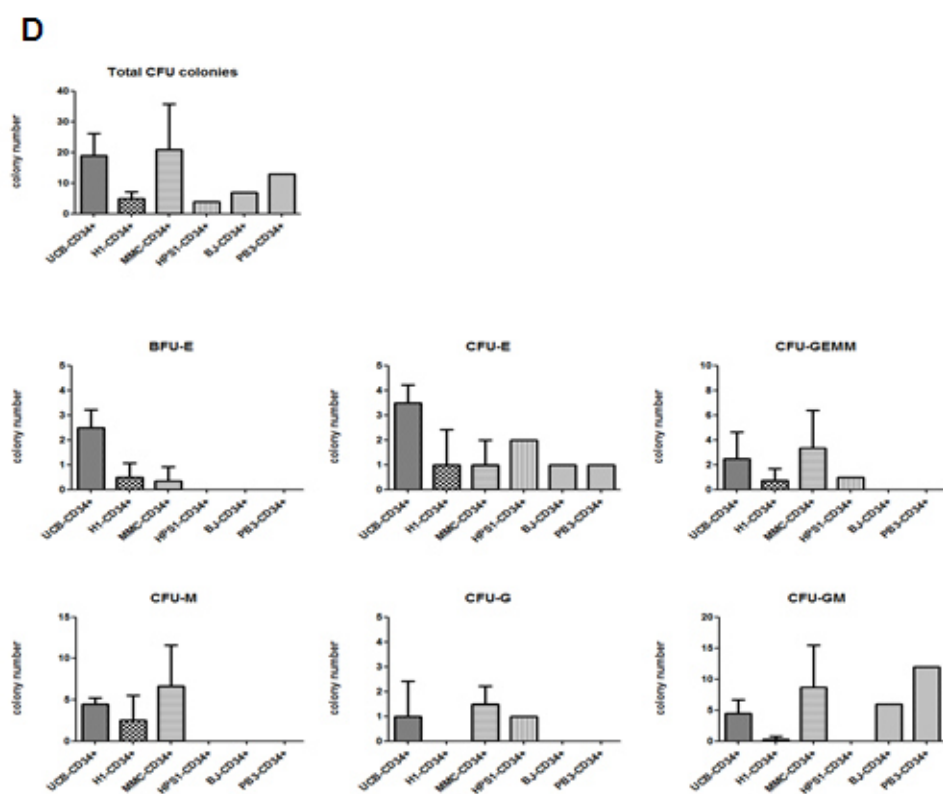
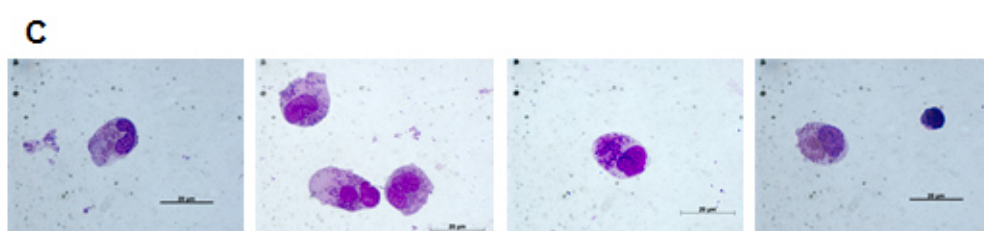
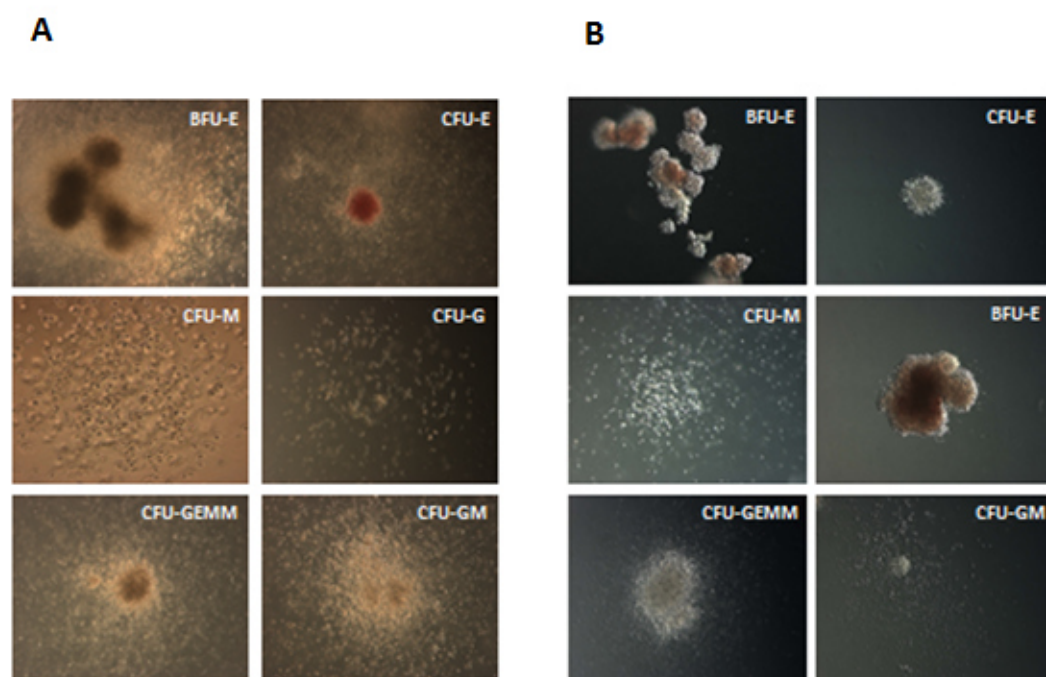
**Figure 3.8 Haematopoietic cell surface marker expression.** Comparison of marker expression in BJ hiPSCs (n=2), BJ hiPSC-derived CD34<sup>+</sup> cells (n=2) and UCB-derived CD34<sup>+</sup> cells (n=2). FACS histograms represent data from the BJ hiPSC line and UCB-derived CD34<sup>+</sup> cells. The blue histogram in each plot shows the isotype control and red histogram specific staining.

### 3.2.7 Assessment of myeloid differentiation potential with colony forming unit (CFU) assays

To assess the *in vitro* differentiation potential of hPSC-derived CD34<sup>+</sup> cells (OP9 co-culture derived) into multi lineage myeloid progenitors, we performed CFU assays with CD34-enriched cells derived from UCB, hiPSC MMC and hESC lines. After 14 days of culture in a semi-solid methylcellulose medium supplemented with cytokines as per the manufacturer's protocol, we determined the total colony number and characterized these colonies by morphology. All hPSC-derived CD34<sup>+</sup> cells from OP9 co-cultures proliferated and formed colonies (Fig. 3.9 A, B). Progenitor cells of

the erythroid lineage were determined as either burst-forming-unit erythrocytes (BFU-E) which are typically >200 early erythrocyte progenitor cells in 3-8 densely packed clusters or colony-forming-unit erythrocyte (CFU-E) which is defined as a packed cluster of 8-200 erythrocyte progenitors. The colour ranged from dark red/orange to brown. Staining with May Grunwald Giemsa (MGG) further confirmed that cells changed their morphology towards developing myeloid and erythroid cells within our differentiation system. The cells have some features of developing myeloid cells including the presence of granules and nuclear morphology resembling metamyelocytes and myelocytes (Figure 3.9 C).

Although all hPSC-derived CD34<sup>+</sup> cells could give rise to CFUs, lower colony numbers could be observed when compared to UCB-derived CD34<sup>+</sup> cells. Limited comparative studies showed that hESC-derived CD34<sup>+</sup> cells formed  $5 \pm 1$  (n=3) colonies. In contrast hiPSC MMC-derived CD34<sup>+</sup> cells gave rise to  $21 \text{ colonies} \pm 15$  (n=3). We observed lower BFU-E and CFU-E in hPSC-derived CD34<sup>+</sup> cells compared to UCB-derived HSCs and it was difficult to subcategorise myeloid colonies. However, our preliminary data suggest a predominant expansion of myeloid CFUs in MMC hiPSC-derived CD34<sup>+</sup> cells compared to UCB-CD34<sup>+</sup> cells.



**Figure 3.9 Myeloid and erythroid progenitors form in CFU assays.** (A) CFU assays with UCB- and (B) hiPSC-derived CD34<sup>+</sup> cells after 14 days of culture. (C) May-Gruenwald Giemsa stain with 100x oil magnification. (D) A comparison of colony numbers shows predominant expansion of myeloid progenitors with limited potential of hiPSC-derived cells to form erythroid progenitor colonies. Results are presented as Mean±SD.

### 3.2.8 Transcriptional control during HSC differentiation in OP9 co-culture

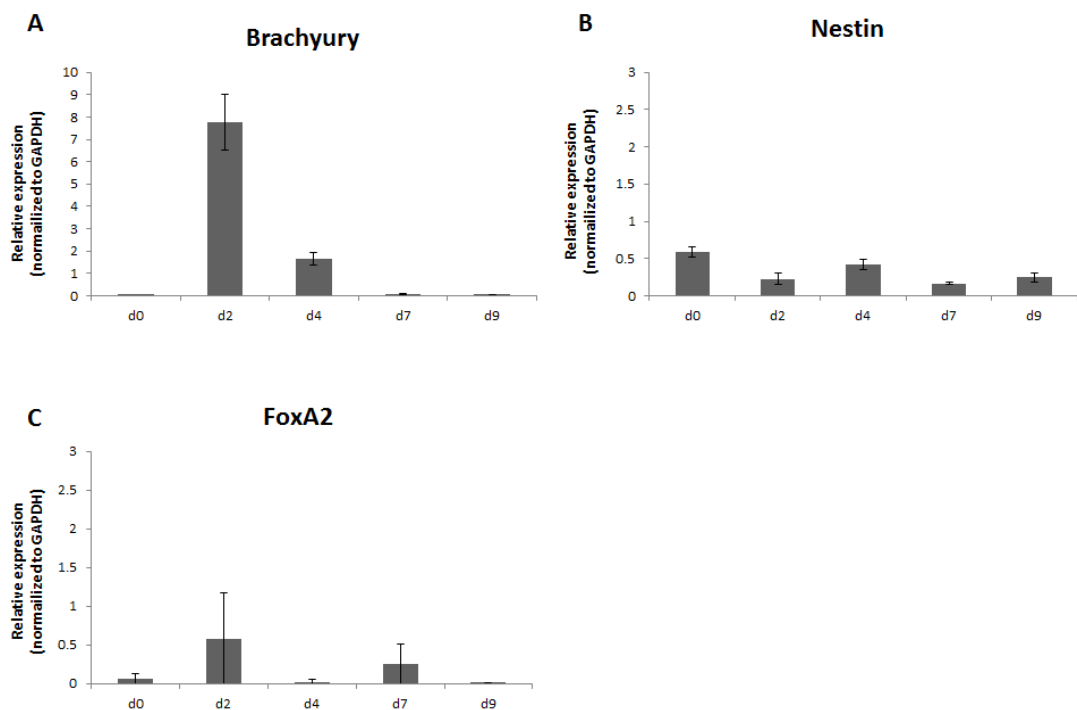
In order to investigate the reason for low efficiencies of CD34<sup>+</sup> cell generation, we decided to perform gene expression analysis by RT-qPCR. We hypothesised, that cells, within the co-culture, differentiate into other lineages than haematopoietic progenitors. For this experiment we collected RNA samples at different timepoints of differentiation (d0 – d9) and designed primers for genes specific for certain cell types and lineages.

#### 3.2.8.1 Genes of the three germ layers

In the first instance, we investigated the expression levels of markers of the three germ layers. We observed high upregulation of the mesodermal marker Brachyury from  $0,0 \pm 0$  (n=3) on day 0 to  $7,8 \pm 1,2$  (n=3) relative expression on day 2 (Fig. 3.10 A). Relative expression significantly decreased again to  $1,7 \pm 0,3$  (n=3) at day 4 and stayed down-regulated towards the end of the co-culture. To further investigate if early ectoderm or endoderm was induced as well, we followed the expression pattern of Nestin and FoxA2, respectively. The relative expression of Nestin was



slightly upregulated from  $0,2 \pm 0,007$  (n=3) on day 2 to  $0,4 \pm 0,07$  (n=3) on day 4 of differentiation (Fig. 3.10 B). These results indicate that early ectoderm is only minimally induced in the OP9 co-culture. No significant upregulation of the endodermal marker FoxA2 could be observed at any time point of the differentiation (Fig. 3.10 C). These data suggest, that the mesodermal lineage is primarily induced in the differentiation with OP9 co-culture.

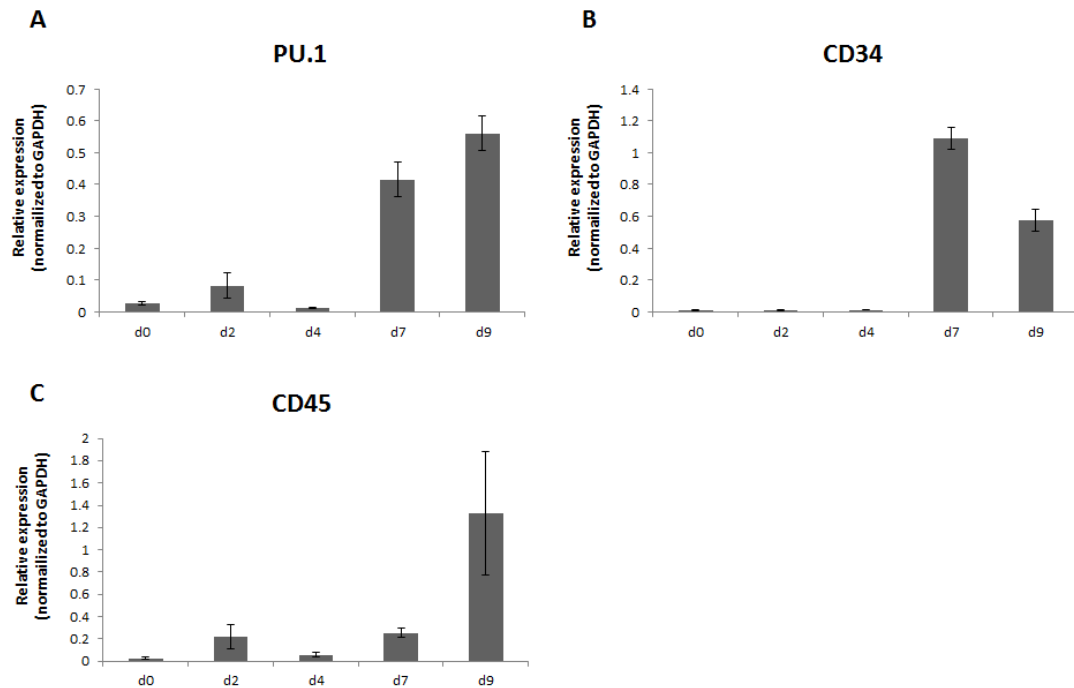


**Figure 3.10 Gene expression of the 3 germ layers.** The relative expression of (A) mesodermal (Brachyury), (B) ectodermal (Nestin) and (C) endodermal (FoxA2) marker genes was determined by RT-qPCR on day 0,2,4,7 and 9 of OP9 co-culture. Relative expression of Brachyury peaked at day 2 with  $7,8 \pm 1,2$  (n=3), which indicates that mainly mesoderm is induced in our differentiation cultures. Results are presented as Mean $\pm$ SD, n=3 technical replicates.

### 3.2.8.2 Haematopoietic genes

To investigate the kinetics of upregulation of haematopoietic genes on the RNA level, we followed the expression levels of haematopoietic-specific genes. The relative expression of PU.1 was upregulated from  $0,01 \pm 0,003$  (n=3) on day 4 to  $0,4 \pm 0,05$  (n=3) on day 7 and to  $0,6 \pm 0,06$  (n=3) on day 9 of co-culture (Fig. 3.11 A). CD34 was also upregulated from  $0,01 \pm 0,001$  (n=3) on day 4 to  $1,1 \pm 0,07$  (n=3) on day 7 and slightly downregulated on day 9 to  $0,6 \pm 0,07$  (n=3) (Fig. 3.11 B).

The relative expression of CD45 was upregulated towards the end of the co-culture from  $0,06 \pm 0,02$  (n=3) on day 4 to  $0,3 \pm 0,04$  (n=3) on day 7 and  $1,3 \pm 0,6$  (n=3) on day 9 (Fig. 3.11 C). RT-qPCR data show, that CD34 is most upregulated at day 7 of co-culture with lower CD45 co-expression than on day 9. These results suggest that the haematopoietic progenitor state might be more immature after 7 days of co-culture.

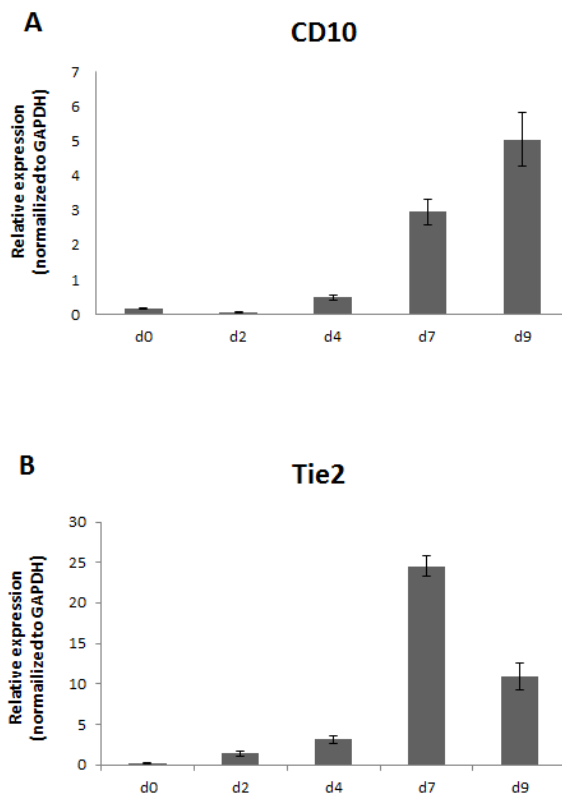


**Figure 3.11 Expression of haematopoietic genes.** The relative expression of the haematopoietic markers (A) PU.1, (B) CD34 and (C) CD45 was determined by RT-qPCR on day 0,2,4,7 and 9 of OP9 co-culture. PU.1 expression was upregulated on day 7 and peaked on day 9 with  $0,6 \pm 0,06$  (n=3). CD34 relative expression was highest on day 7 with  $1,1 \pm 0,07$  (n=3), whereas the relative expression of CD45 was highest on day 9 with  $1,3 \pm 0,6$  (n=3). Results are presented as Mean $\pm$ SD, n=3 technical replicates.

### 3.2.8.3 Mesenchymal and endothelial genes

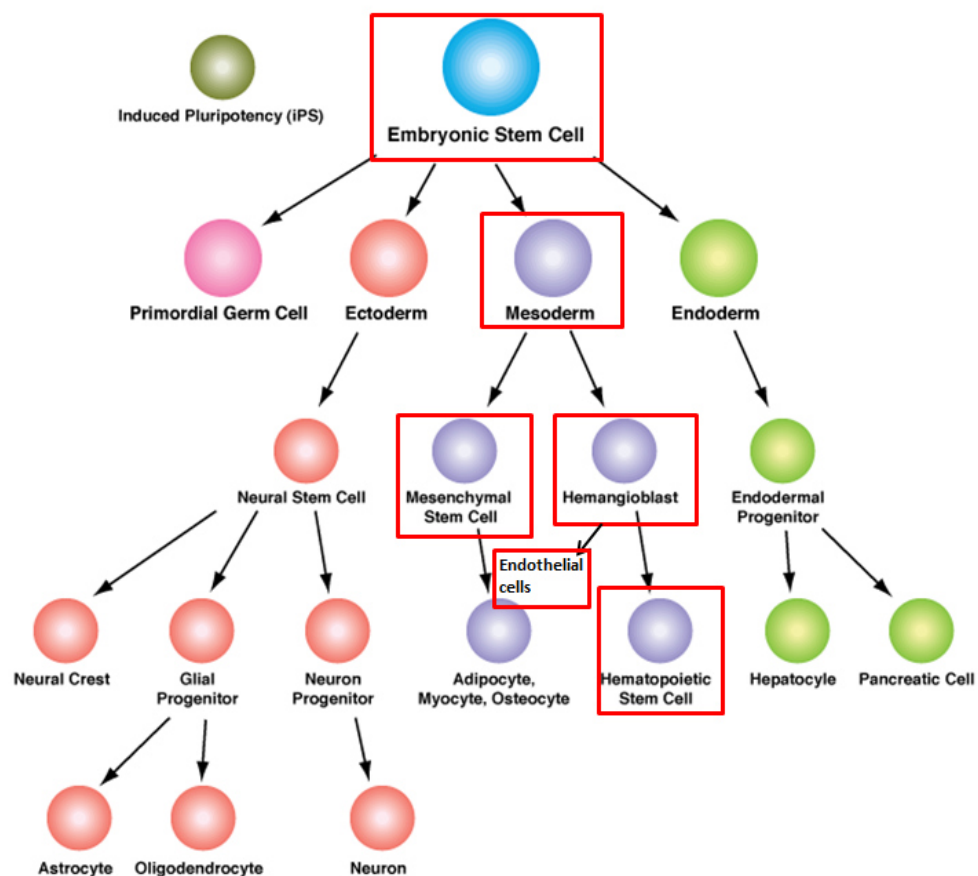
The relative expression of the mesenchymal marker CD10 was steadily upregulated from  $0,05 \pm 0,008$  (n=3) on day 4 to  $2,9 \pm 0,4$  (n=3) on day 7 and up to  $5,0 \pm 0,8$  (n=3) on day 9 of co-culture (Fig. 3.12 A). These results strongly indicate that mesodermal precursor cells not only differentiate into the haematopoietic lineage, but also into mesenchymal progenitors. Furthermore, relative expression of the

early endothelial marker Tie2 highly increased from  $3,2 \pm 0,5$  (n=3) on day 4 to  $24,5 \pm 1,2$  (n=3) on day 7 and decreased again to  $10,9 \pm 1,7$  (n=3) on day 9 (Fig. 3.12 B). The strong increase in relative expression of the endothelial lineage occurred at the same time points (day 7 onwards) as the haematopoietic marker up-regulation. These results support reports in the literature of parallel development of haemato-endothelial precursor cells.



**Figure 3.12 Expression of mesenchymal and endothelial genes.** The relative expression of the (A) mesenchymal marker CD10 and (B) early endothelial marker Tie2 was determined by RT-qPCR on day 0,2,4,7 and 9 of OP9 co-culture. CD10 was upregulated from  $0,05 \pm 0,008$  (n=3) on day 4 to  $5,0 \pm 0,8$  (n=3) on day 9. Relative expression of the endothelial marker Tie2 peaked at day 7 with  $24,5 \pm 1,2$  (n=3), suggesting parallel development of endothelial and haematopoietic progenitor cells. Results are presented as Mean $\pm$ SD, n=3 technical replicates.

The transcriptional analysis of lineage-specific genes showed a predominant mesoderm induction within the first two days of HSC differentiation. The upregulation of haematopoietic markers could be confirmed, but also upregulation of mesenchymal, endodermal and endothelial genes. These data suggest that the co-culture on OP9 feeder cells does not only support differentiation towards the haematopoietic lineage but into a variety of progenitor cells. This results in a very heterogeneous population, including mesenchymal and endothelial cells – which can express CD34 on their cell surface.



**Figure 3.13 A model of lineage differentiation from the pluripotent stem cell state.** The first specification of haematopoietic development is mesodermal

differentiation, followed by the formation of the haemangioblast and haemato-  
endothelial progenitors, where haematopoietic stem cells derive from.

### 3.3 Discussion

In this chapter we investigated if GMP compliant, integration-free hiPS cell lines have the potential to differentiate to haematopoietic progenitor cells. Although the differentiation of hiPSC to different haematopoietic lineages has been proven *in vitro* in various studies (Ackermann, Liebhaber et al. 2015), the generation of fully defined haematopoietic progenitors remains challenging and inefficient. Furthermore, HSCs with engraftment and reconstitution potential have not been generated with *in vitro* protocols so far.

We established a protocol to generate haematopoietic progenitors from hPSCs in our lab from scratch. Various differentiation approaches are published in the literature and the differentiation with OP9 feeder cells proved to be the most robust and reproducible in our hands. We tested four different hiPS cell lines derived from fibroblasts, one hiPS cell line derived from PBMCs and the H1 hES cell line. All cell lines did undergo typical morphological changes within nine days of OP9 co-culture. CD34 expression varied between cell lines and in different experiments with the same cell line. Interestingly, we observed slightly higher CD34 efficiencies with hiPSCs compared to hESCs. The observation, that hiPSCs derived from PBMCs differentiated more efficiently towards the haematopoietic lineage was interesting and lead to the question, if the efficiency is significantly influenced by the origin of the reprogrammed cells. The epigenetic memory of cells - where DNA methylation signature remains present in hiPSCs depending on their somatic origin and influences the differentiation ability towards specific lineages - has been described in the literature and could explain our observation (Kim, Doi et al. 2010). Although the derivation of CD34-expressing cells remained low, our data were comparable to what was published in the literature and the protocol was robust and reproducible.

The CD34-expressing cell fraction could be enriched with magnetic activated cell sorting and was phenotypically compared to UCB-derived CD34<sup>+</sup> cells.

A fraction of derived CD34<sup>+</sup> cells co-expressed the haematopoietic markers CD43 and CD45 to comparable levels in all cell lines. Our data show that after CD34 enrichment, 90% of cells are of human origin as assessed by Tra-1-85 staining, so most OP9 cells are depleted in this enrichment step. The pluripotency markers Tra-1-60 and Tra-1-81 are downregulated during OP9 co-culture and are not expressed after 9 days of differentiation. We further compared the phenotype of hiPSCs, enriched hiPSC-derived CD34<sup>+</sup> and UCB-derived CD34<sup>+</sup> cells by surface marker expression with flow cytometry. Interestingly, when we compared immunophenotypes of enriched hESC-derived with UCB-CD34<sup>+</sup> cells we observed distinct CD34<sup>dim</sup> and CD34<sup>bright</sup> populations in hESC-derived cells. These findings are consistent with reports in the literature, where Dravid et al. identified two subpopulations within hESC-derived CD34<sup>+</sup> cell compartment with either haemato-endothelial (CD34<sup>bright</sup>) or haematopoietic-restricted (CD34<sup>dim</sup>) potential (Dravid, Zhu et al. 2011). Furthermore, our data suggest that CD43 is co-expressed by CD34<sup>dim</sup> cells, which indicates that this cell population represents cells with haematopoietic potential. Further characterisation of the CD34<sup>dim</sup> and CD34<sup>bright</sup> populations in regards to their haematopoietic differentiation potential would have been of interest. CD45 is co-expressed on all CD34<sup>+</sup> cells of UCB-derived HSCs (data not shown) and we observed expression of this marker within the CD34<sup>dim</sup> population of hESC-derived cells. Altogether these data suggest that only a small fraction of isolated hPSC-derived CD34<sup>+</sup> cells are committed to the haematopoietic lineage.

CD34, CD43 and CD45 are not expressed in hiPSCs and are upregulated during OP9 co-culture. With CD34 MACS enrichment we could achieve purities of more than 90%, although in some experiments the purity was significantly lower.



Interestingly, CD43 is used as a marker to identify haematopoietic precursors in most studies differentiating HSCs from hiPSCs (Knorr, Ni et al. 2013) (Woll, Martin et al. 2005) (Vodyanik, Bork et al. 2005) (Choi, Vodyanik et al. 2011). However, CD43 is not expressed on UCB-derived CD34<sup>+</sup> cells. The pan leukocyte marker CD45 is expressed on all UCB-derived CD34<sup>+</sup> cells but only in variable fractions in hiPSC-derived CD34<sup>+</sup> cells. The stem cell markers CD117 and CD133 are expressed on hiPSCs and our data suggest downregulation in the enriched CD34<sup>+</sup> cell population compared to higher expression in UCB-derived CD34<sup>+</sup> cells. Interestingly, we observed CD56 expression in hiPSCs which is downregulated in hiPSC-derived CD34<sup>+</sup> cells and not expressed in UCB-derived CD34<sup>+</sup> cells. We further investigated, if hiPSC-derived CD34<sup>+</sup> cells exhibit the ability to differentiate into more mature haematopoietic cells in an *in vitro* colony forming unit assay. The enriched CD34<sup>+</sup> cells successfully generated myeloid and erythroid progenitors with distinct morphological appearance. However, we observed lower colony numbers with hiPSC-derived cells compared to UCB-derived cells, especially erythroid progenitor colonies. This observation is probably due to lower numbers of actual haematopoietic progenitor cells within the hPSC-derived CD34 population compared to UCB-derived CD34<sup>+</sup> cells. Furthermore, hPSC-derived cells might exhibit a more fetal phenotype of HSCs and therefore might not have the same differentiation potential. The *in vivo* engraftment into immunocompromised mice and reconstitution of the entire haematopoietic system would be the ultimate functional test for hPSC-derived haematopoietic stem cells.

Beside the OP9 co-culture method, we explored clinically applicable methods using a serum- and feeder free approach with APEL medium. Although CD34-expressing cells could be generated with this method, the derived CD34<sup>+</sup> cells did not co-express either CD43 or CD45. Furthermore, these cells did not form myeloid or erythroid colonies in CFU assays. We further explored differentiation with embryoid

body (EB) formation which has been shown to provide an environment closer to the *in vivo* development than 2D cultures, resulting in higher efficiencies of HSCs (Ng, Davis et al. 2008) (Knorr, Ni et al. 2013). Although EBs could successfully be derived from hESCs and hiPSCs and were cultured under mesoderm/haematopoietic-inducing conditions, the dissociation into a live single cell suspension could not be achieved in our hands. EBs are very compact, three-dimensional aggregates with complex cell-adhesions.. Due to this problem we could not analyse the marker expression via FACS and could not enrich the haematopoietic precursors with magnetic beads. Several enzymes for dissociation were tested, including Accutase, trypsin/EDTA, tryPLE and gentle dissociation reagent. To improve the dissociation step in this protocol an “Embryoid body dissociation kit” could be tested in future experiments available from Miltenyi Biotec. Furthermore, the density of formed EBs can critically affect the successful dissociation. For the spin EB method we have used 3000 pluripotent cells/EB where cell numbers could be down-scaled to avoid the formation of highly compact EBs, although this might have a negative effect differentiation efficiencies.

Further studies with hiPSC-derived CD34<sup>+</sup> cells revealed lower haematopoietic differentiation potential compared to UCB-derived CD34<sup>+</sup> cells, tested in CFU assays and NK cell differentiation protocols (discussed in Chapter 4). Although CD34 is commonly used as a haematopoietic stem cell marker, it is also expressed on endothelial and mesenchymal cells. To address the potential problem that CD34-expressing cells generated with the established OP9 co-culture are primed towards the haematopoietic or other lineages, we performed gene expression analysis with cells derived with OP9 co-culture differentiation.

Our data suggest that mainly the mesodermal lineage is induced within the first two days of differentiation which represents the first specification of haematopoietic development from PSCs. The haematopoietic markers PU.1, CD34 and CD45 are upregulated during differentiation indicating the successful derivation of haematopoietic progenitors within the culture. Interestingly, the mesenchymal marker CD10 and endothelial marker Tie2 are highly upregulated beside haematopoietic markers. These data suggest that OP9 co-culture does not solitary support differentiation towards the haematopoietic lineage, but also generates mesenchymal and endothelial progenitor cells. We believe, that this explains the low efficiencies in the differentiation to terminal haematopoietic cells.

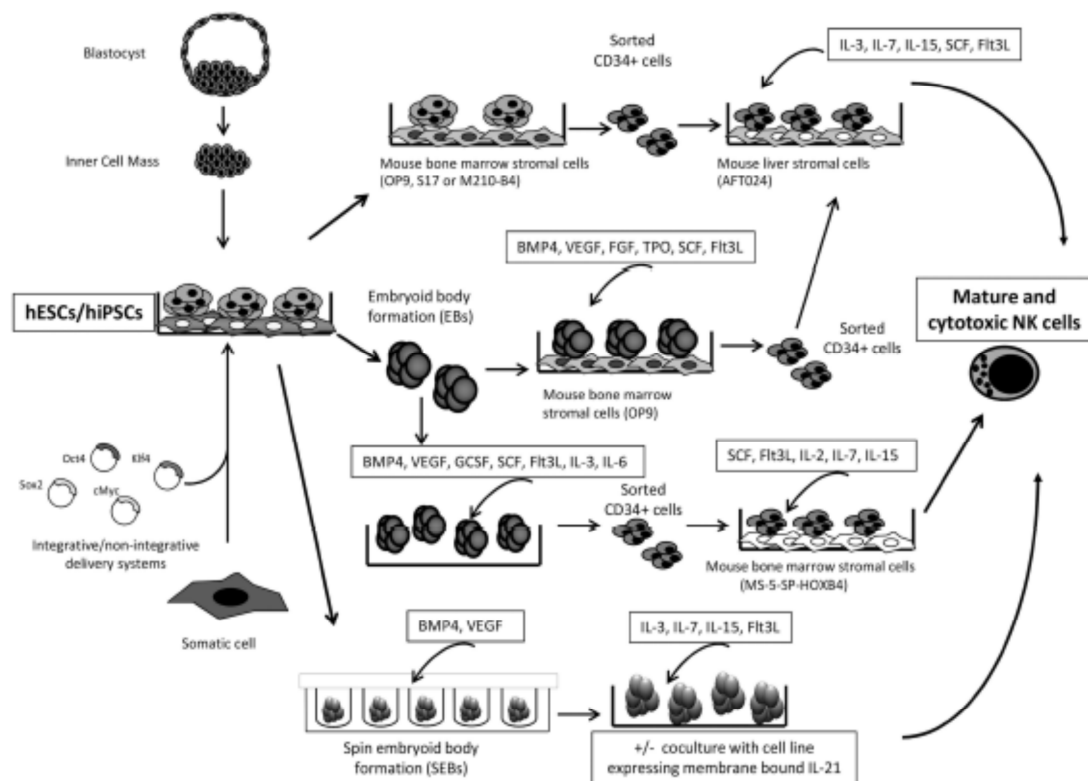
## Chapter 4 Generation of hiPSC- derived NK cells and their characterisation

### 4.1 Introduction

Haematopoietic progenitors are a promising source for the large scale generation of functional and pure allogeneic NK cells. Various studies describe the differentiation of large numbers of functional NK cells from umbilical cord blood (UCB)-derived CD34<sup>+</sup> haematopoietic progenitor cells with co-culture systems in combination with cytokines (Frias, Porada et al. 2008; Pinho, Punzel et al. 2011; Larbi, Gombert et al. 2012; Luevano, Madrigal et al. 2012; Luevano, Domogala et al. 2014). Furthermore, the generation of allogeneic NK cells without feeder cells and under GMP conditions opened doors for clinical trials (Spanholtz, Tordoir et al. 2010; Spanholtz, Preijers et al. 2011). These cells have shown efficient killing of bone marrow-residing human leukemia cells in NOD/SCID/IL2Rg(null) mice (Cany, van der Waart et al. 2013). Further studies with these *in vitro* culture conditions revealed that the addition of IL-12 can direct the NK cell differentiation towards more mature NK cells with enhanced properties (Lehmann, Spanholtz et al. 2014).

HPSCs could represent an unlimited source of NK cells enabling administration of high cell doses for immunotherapy and supporting the development of a well characterized repository of potentially “off the shelf” cells. The differentiation of hPSCs into functional NK cells has been described in the literature and although varying efficiencies in haematopoietic development can be observed in different hESC and hiPSC lines, functional NK cells can be effectively generated (Figure 4.1) (Woll, Martin et al. 2005; Knorr, Ni et al. 2013) (Vodyanik, Bork et al. 2005; Woll, Morris et al. 2008; Raya, Rodriguez-Piza et al. 2009; Ni, Knorr et al. 2011; Larbi, Gombert et al. 2012; Knorr, Bock et al. 2013).

Derivation methods are based on a two-step differentiation protocol. The first important step is the generation of CD34<sup>+</sup> haematopoietic precursors from PSCs, discussed in Chapter 3. At this stage most protocols require cell sorting or enrichment of HSCs due to low efficiencies in the differentiation process. In the second step, PSC-derived HSCs are cultured either on stromal cell lines or in differentiation medium supplemented with cytokines to promote NK cell differentiation. Woll et al. described the co-culture of hESC-derived CD34<sup>+</sup> cells with murine fetal liver-derived AFT024 stromal cells in a medium supplemented with IL-15, IL-3, IL-7, SCF and fms-like tyrosine kinase receptor-3 ligand (Flt3L) (Woll, Martin et al. 2005). After 30 days of culture, cells expressed NK cell maturation markers including NCRs, CD16, CD94/NKG2A and KIRs and cells could efficiently lyse malignant cells (Woll, Martin et al. 2005). Co-culture on the mouse fetal liver cell line EL08.1D2 in combination with IL-3, IL-7, IL-15, SCF and Flt3L has been described in the literature and will be discussed in more detail in the result chapter below. Another approach utilises the co-culture of CD34<sup>+</sup> haematopoietic progenitors on OP9 stromal cells in combination with the following cytokines: BMP4, VEGF, SCF, FGF, TPO and Flt3L (Raya, Rodriguez-Piza et al. 2009).



**Figure 4.1 Schematic overview of NK cell derivation methods from hPSCs.**

Figure adapted from (Eguizabal, Zenarruzabeitia et al. 2014).

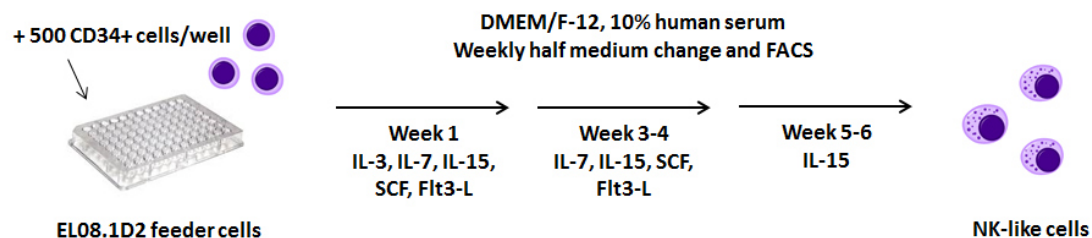
A clinical-scale derivation protocol of NK cells from hPSCs was published by Knorr et al. (Knorr, Ni et al. 2013), generating functional NK cells with the spin EB method in the absence of cell sorting and without the support of stromal cell lines, suggesting that hPSC-derived progenitors may produce their own stromal support. This study reports that NK cells can be generated at high numbers from both, hESCs and hiPSCs, achieved by a final expansion on artificial antigen-presenting cells (aAPCs) expressing membrane bound IL-21. Generated cells express NK cell-specific surface markers including CD94, KIR, CD16, NKG2D and NKp46 in comparable level to peripheral blood and UCB-derived NK cells. hPSC-derived NK cells furthermore show efficient killing of the leukemic cell line K562 in an *in vitro*

killing assay (Knorr, Ni et al. 2013). NK cells generated with this method further show efficacy against leukemia, HIV infected cells and ovarian cancer in cellular assays and animal models (Ni, Knorr et al. 2011) (Hermanson, Bendzick et al. 2016).

## 4.2 Results

### 4.2.1 NK cell derivation from hPSC-derived CD34<sup>+</sup> cells with the mouse fetal liver cell line EL08.1D2 co-culture system

The aim of this study was to test the ability of hPSC-derived CD34<sup>+</sup> cells (described in chapter 3) to acquire NK cell-specific surface marker expression. We adapted a protocol from collaborators at the Royal Free Hospital which facilitates successful differentiation of UCB-derived CD34<sup>+</sup> cells into functional NK cells (Grzywacz, Kataria et al. 2006) (Luevano, Madrigal et al. 2012) (Luevano, Domogala et al. 2014). The method is based on a co-culture system using the mouse fetal liver cell line EL08.1D2 in combination with the following cocktail of cytokines: IL-3 (week one only), IL-15, IL-7, SCF in weeks 1-4 and IL-15 only for weeks 5 and 6 (Figure 4.2).

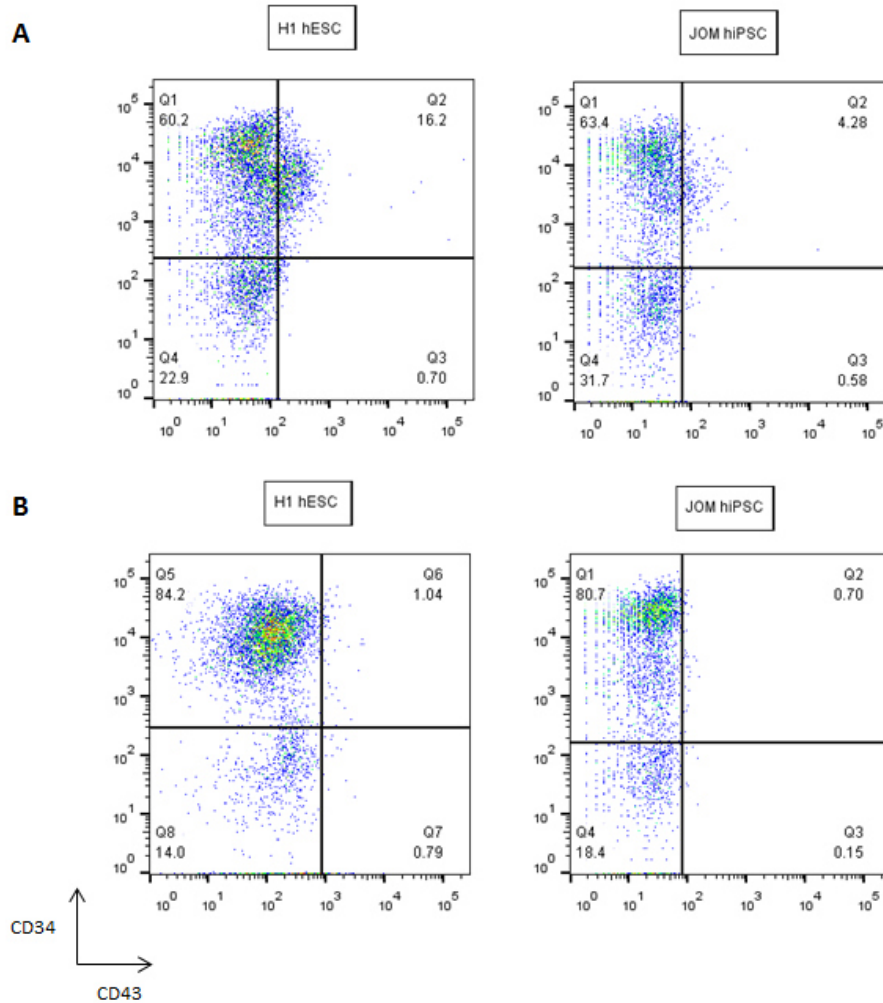


**Figure 4.2 Schematic overview of the EL08.1D2 co-culture protocol for NK cell differentiation *in vitro*.** CD34<sup>+</sup> cells are co-cultured with the mouse fetal liver cell line EL08.1D2 for five to six weeks, supplemented with cytokines to induce NK cell differentiation.



#### **4.2.2 HPSC-derived CD34<sup>+</sup> differentiate into CD56<sup>+</sup>CD3<sup>-</sup> NK like cells**

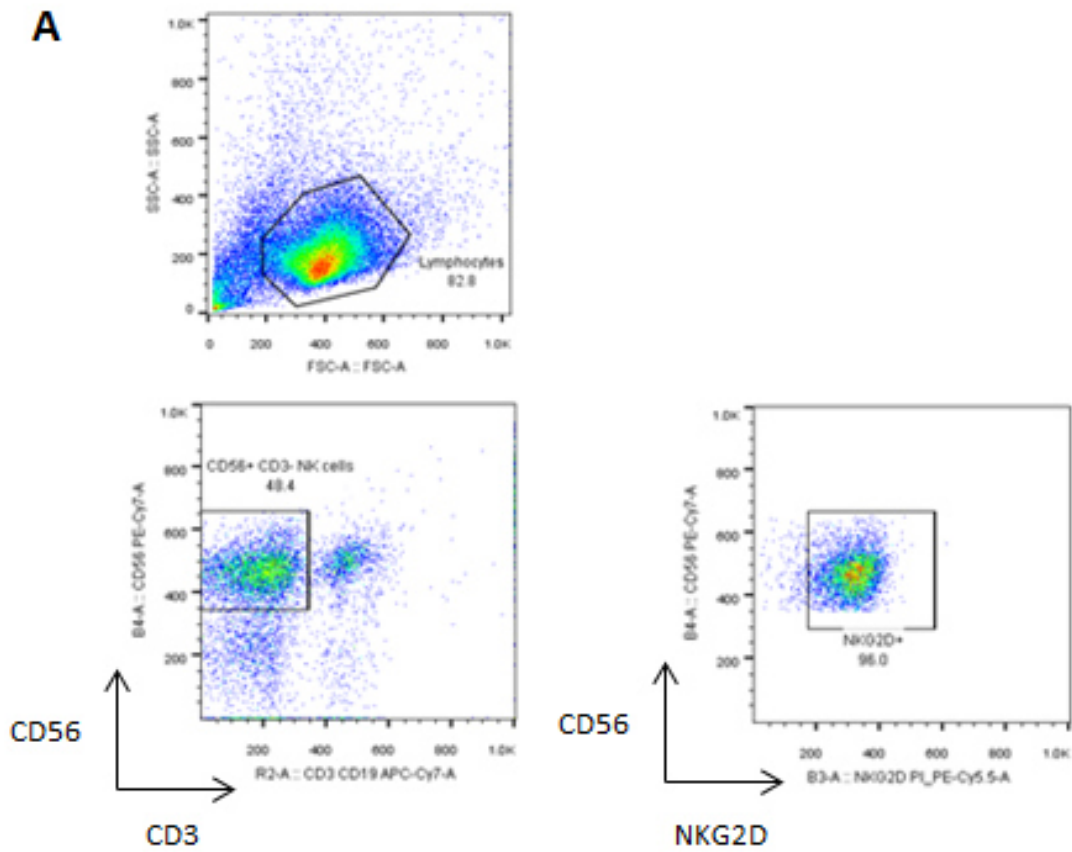
The initial experiment of this study was performed in our collaborators laboratory where the protocol was established to generate large numbers of NK cells from UCB-derived CD34<sup>+</sup> cells. We tested hESC- and hiPSC-derived CD34<sup>+</sup> cells previously derived with two different methods: OP9 co-culture and APEL differentiation (described in chapter 3). CD34<sup>+</sup> MACS enriched cells - derived with OP9 co-culture - from hESC H1 were used at a purity of 76% CD34<sup>+</sup> cells from which 16% co-expressed CD43. JOM hiPSC-derived cells were used at a purity of 67% CD34<sup>+</sup> cells with 4% co-expressing CD43 (Figure 4.3 A). CD34<sup>+</sup> MACS enriched cells derived with the APEL differentiation method were used at a purity of 85% for H1 hESCs and 81% for JOM hiPSCs (Figure 4.3 B). Co-expression of CD43 could not be observed in these cells which correlates with findings discussed in Chapter 3. Cells were seeded onto irradiated EL08.1D2 feeder cells at 2000 cells/96-well. The protocol is optimised to seed 500 UCB-derived CD34<sup>+</sup> cells/well EL08.1D2 feeder cells which will expand to up to 1x10<sup>6</sup> NK cells (Grzywacz, Kataria et al. 2006; Luevano, Domogala et al. 2014). For this initial experiment we decided to plate higher cell numbers/well because of lower purity of CD34-expressing cells. Cell numbers limited this experiment to one well for each H1 hESC sample (OP9 co-culture and APEL derived), two wells for JOM hiPSC APEL derived and two wells for JOM hiPSC OP9 co-culture derived.

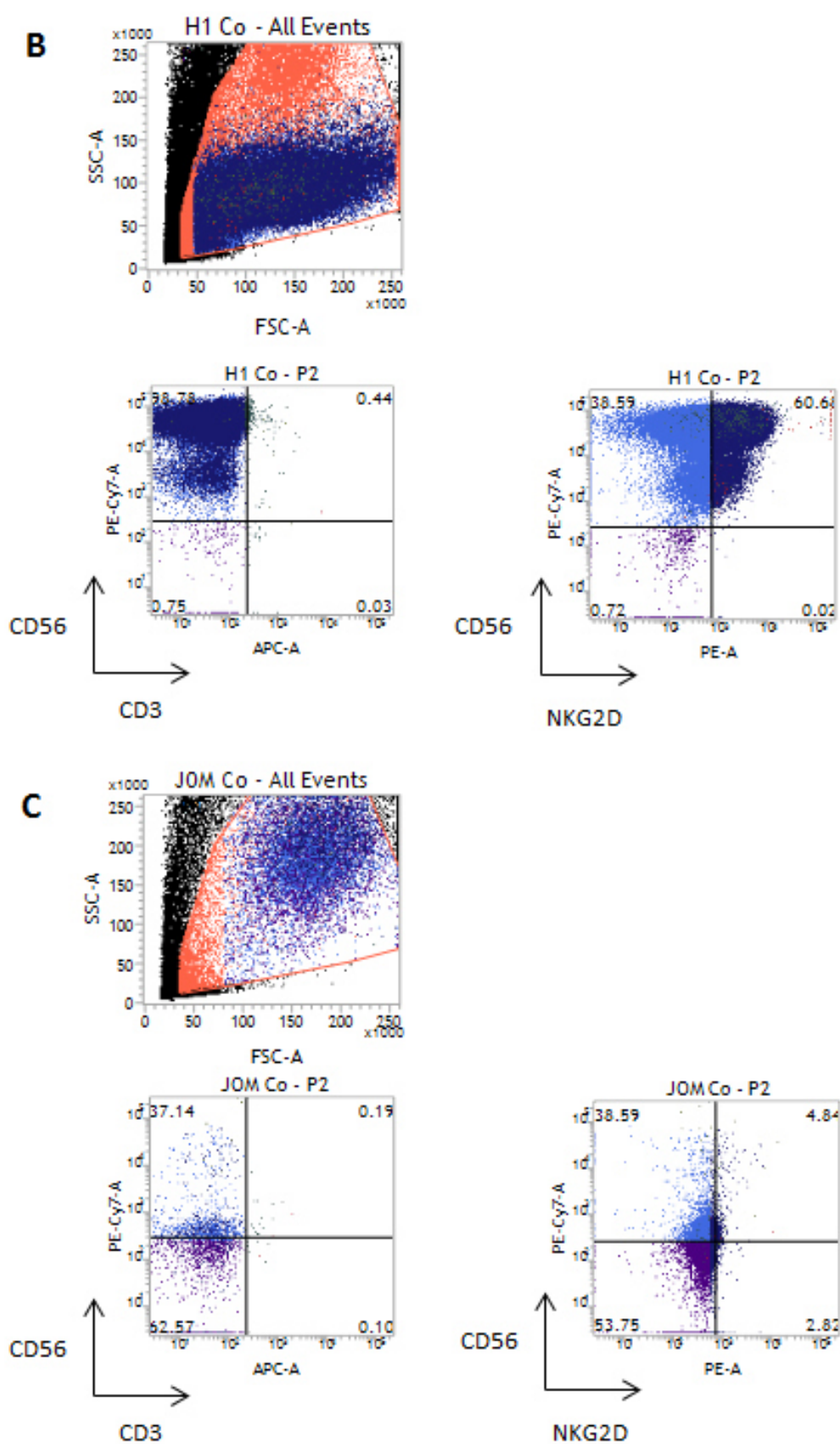


**Figure 4.3 hPSC-derived CD34<sup>+</sup> cells for NK cell differentiation on EL08.1D2 co-culture.** (A) OP9 co-culture derived H1 hESC-derived CD34<sup>+</sup> cells display 76% purity with 16% CD43 co-expression. JOM hiPSC-derived CD34<sup>+</sup> cells from OP9 co-culture with 67% purity and 4% CD43 co-expression. (B) APEL derived H1 hESC- and JOM hiPSC-derived CD34<sup>+</sup> cells display purities of 85% and 81%, respectively without CD43 co-expression.

Cells were cultured following the protocol with weekly medium changes supplemented with respective cytokines for each week (Figure 4.1). Importantly, on day 29 of co-culture, the viability of the cell culture was good and 99% of H1 hESC-

derived cells (from OP9 co-culture) expressed CD56 in the absence of CD3, which characterises the NK cell population (Kiessling, Klein et al. 1975; Lanier, Phillips et al. 1986) (Figure 4.4 A). Furthermore, CD56<sup>dim</sup> and CD56<sup>bright</sup> sub-populations could be observed, with a predominant CD56<sup>bright</sup> NK cell population generated. Due to low cell numbers and the lack of an established multi-colour flow cytometry panel in this initial experiment, we could not stain for a range of NK cell-specific surface markers. However, the activating receptor NKG2D was expressed in 60% of CD56<sup>+</sup> cells.

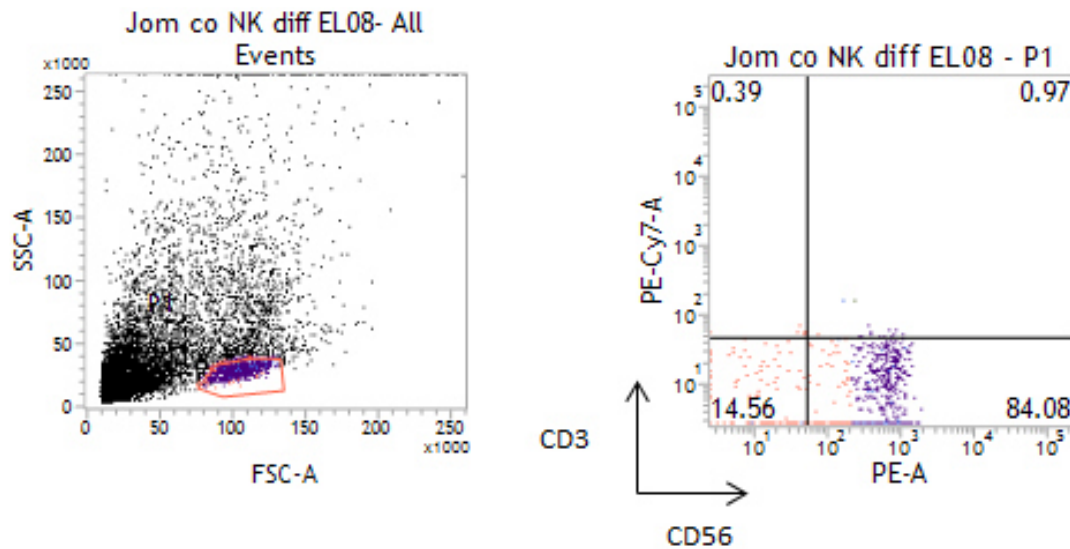




**Figure 4.4 Day 29 NK cell differentiation with hPSC-derived CD34<sup>+</sup> cells (OP9 co-culture derived).** (A) Primary NK cells isolated from fresh blood with negative selection beads. NKG2D expression levels on CD56<sup>+</sup>CD3<sup>-</sup> gated NK cells (B) H1 hESCs display NK cell characteristic expression of 99% CD56, with distinguishable CD56<sup>dim</sup> and CD56<sup>bright</sup> sub-populations. 60% of CD56<sup>+</sup> cells co-expressed the activating NK cell marker NKG2D (C) JOM hiPSC display a more granulated phenotype in the FSC/SSC profile and only 37% of cells express CD56 with 4% NKG2D co-expression. All gates were set based on corresponding isotype controls.

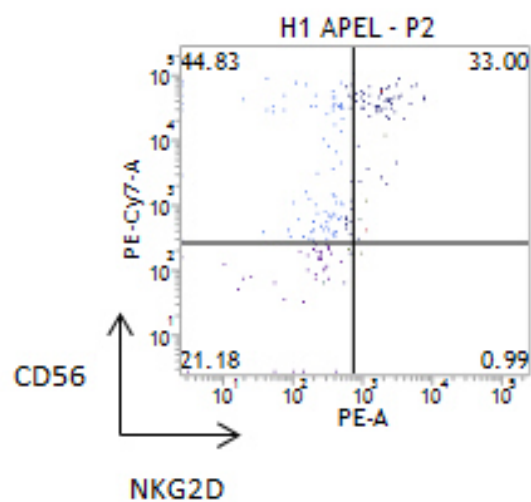
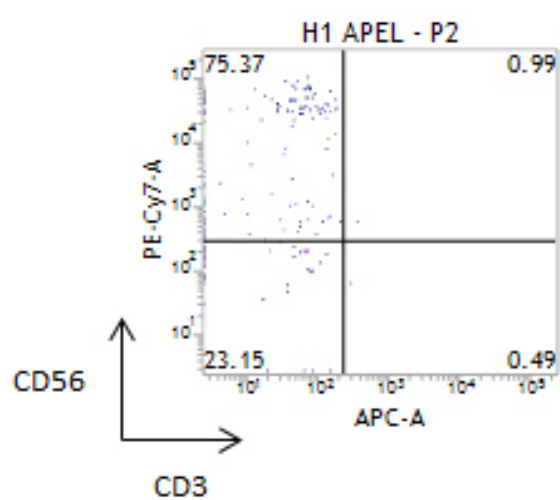
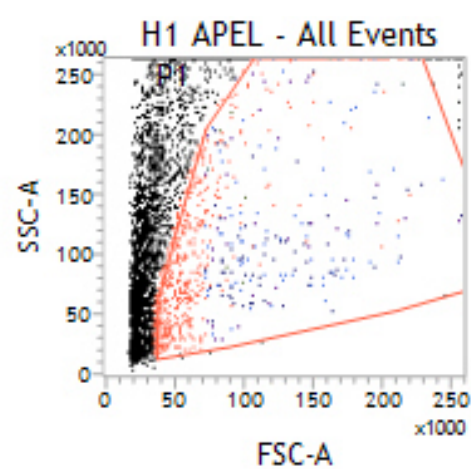
Interestingly, JOM hiPSC-derived cells exhibited a different FSC/SSC profile compared to H1 hESC-derived cells on day 29 of co-culture with cells possessing higher granularity (Figure 4.4 B). CD56 up-regulation could be observed in 37% of cells but no clear separation of CD56<sup>dim</sup> and CD56<sup>bright</sup> sub-populations. Expression of NKG2D could be observed in 4% of CD56<sup>bright</sup> expressing cells.

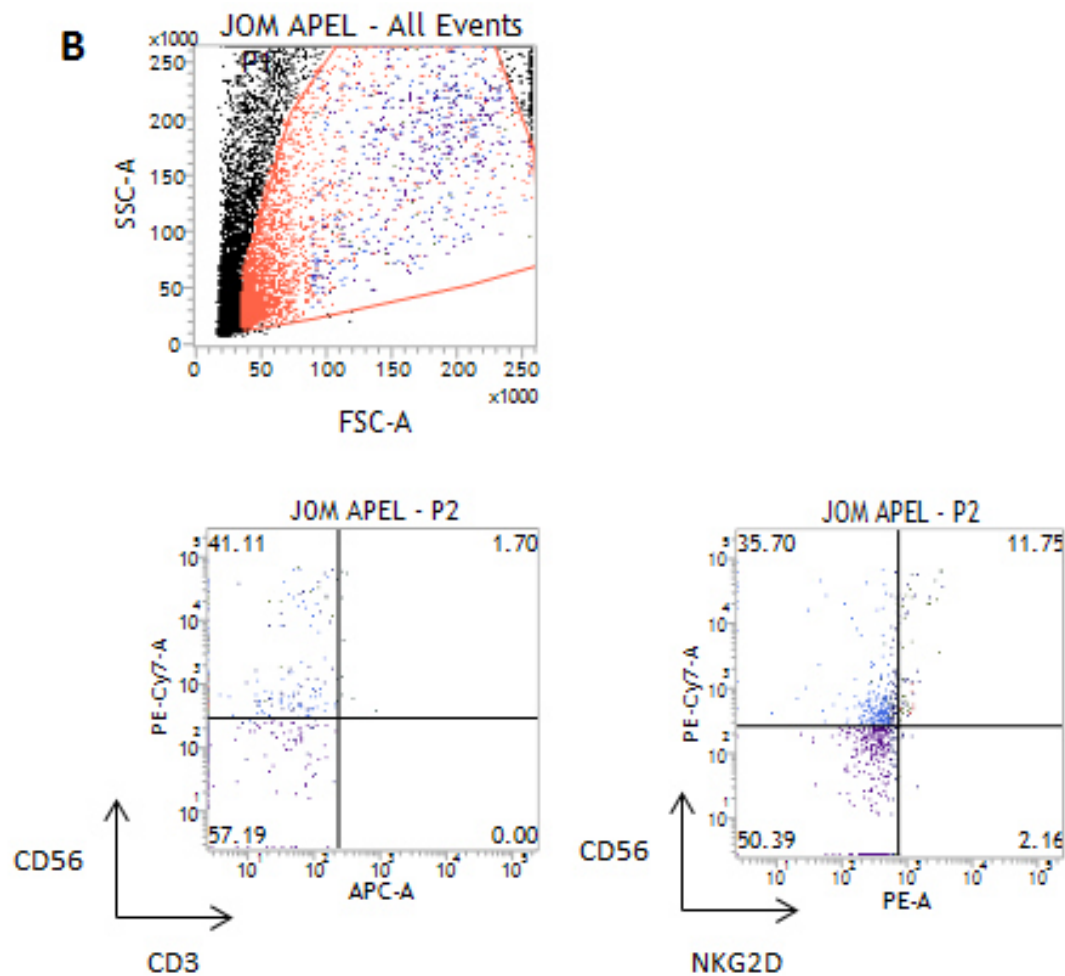
To investigate if hiPSC-derived cells will up-regulate CD56 to a level resembling the H1 hESC-derived NK-like cells, we co-cultured the cells for a further 20 days on EL08.1D2 feeder cells. On day 49, cell numbers and viability was low, however, 84% of JOM hiPSC-derived cells were CD56<sup>+</sup>CD3<sup>-</sup> (Figure 4.5). No distinct sub-populations of CD56<sup>dim</sup> and CD56<sup>bright</sup> could be observed. These data could indicate the presence of less mature progenitors in the JOM hiPSC-derived cell culture on day 29 compared to H1 hESC-derived cells. It might suggest that cells are in a different developmental stage from the beginning of the co-culture or hiPSCs might not have the same differentiation potential as hESCs.



**Figure 4.5 Day 49 NK cell differentiation with JOM hiPSC-derived CD34<sup>+</sup> cells (OP9 co-culture derived).** Low cell numbers and viability were observed after 49 days of EL08.1D2 co-culture, however, 84% of cells were CD56<sup>+</sup>CD3<sup>-</sup>.

We further tested the NK cell differentiation potential of APEL derived CD34<sup>+</sup> cells. The overall viability of APEL derived cells was low after 29 days of EL08.1D2 co-culture when compared to OP9 co-culture derived cells (Figure 4.6). In the small viable cell population we could observe that 75% of H1 hESC-derived cells expressed CD56. No CD3 co-expression could be detected and 33% of cells co-expressed NKG2D (Figure 4.6 A). JOM hiPSC-derived cells revealed a similar FSC/SSC profile compared to OP9 co-culture derived JOM hiPSC with more granulated cells compared to H1 hESC-derived cells. The viability was very low and analysis on the live cell population reveals 42% of cells express CD56 with 11% co-expressing NKG2D (Figure 4.6 B). Further analysis of CD56 up-regulation at a later time point of EL08.1D2 co-culture could not be conducted because of poor cell survival.





**Figure 4.6 Day 29 NK cell differentiation with hPSC-derived CD34<sup>+</sup> cells (APEL derived).** (A) H1 hESCs have a low viability in EL08.1D2 co-culture and display NK cell characteristic expression of 75% CD56, with no clear CD56<sup>dim</sup> and CD56<sup>bright</sup> sub-populations. 33% of CD56<sup>+</sup> cells co-expressed the activating NK cell marker NKG2D (B) JOM hiPSC display a more granulated phenotype in the FSC/SSC profile and only 42% of cells express CD56 with 11% NKG2D co-expression. All gates were set based on corresponding isotype controls.

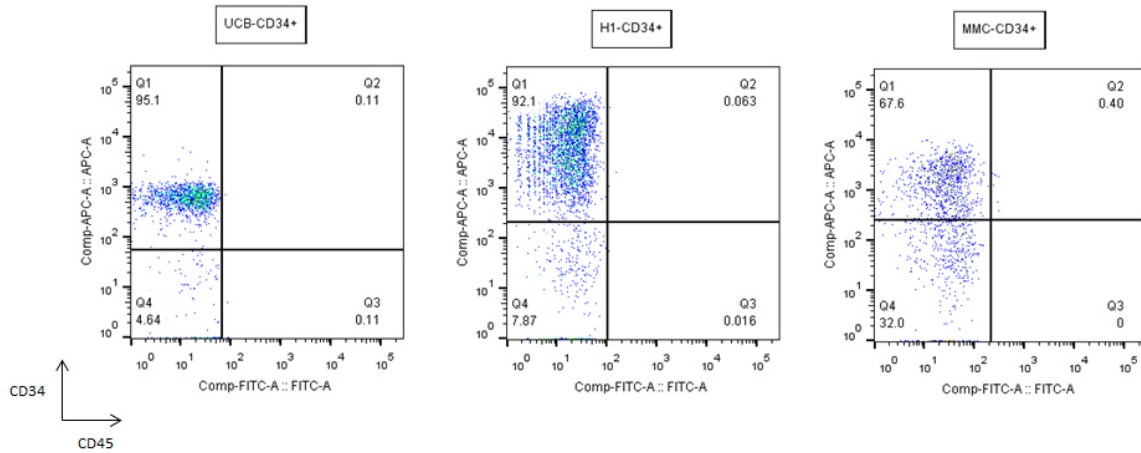
The results of this initial study looked very promising for the prospect to generate NK-like cells from hiPSCs which were generated in our laboratory under GMP-



compliant conditions. Although the overall cell survival and expansion was poor, CD56<sup>+</sup>CD3<sup>-</sup>NKG2D<sup>+</sup> cells could be generated. Data suggest that OP9 co-culture derived CD34<sup>+</sup> cells are more robust for further NK cell differentiation and we decided to conduct further experiments with these cells only.

#### **4.2.3 Immunophenotypic characterisation of UCB- and hPSC-derived CD34<sup>+</sup> cells as starting cell population for NK cell differentiation**

We aimed to establish the EL08.1D2 co-culture protocol for NK cell differentiation in our laboratory, comparing the NK cell generation potential of UCB-, H1 hESC- and MMC hiPSC-derived CD34<sup>+</sup> cells. hPSC-derived CD34<sup>+</sup> cells were generated with OP9 co-culture and CD34<sup>+</sup> cells of all three cell sources were enriched with CD34 MACS technology. The purities of CD34 cultures used in this experiment were 68%, 92% and 95% for MMC hiPSC-, H1 hESC- and UCB-derived cells respectively (Figure 4.7). The purity of MMC hiPSC-derived cells was low and ideally should have been enriched further. The CD45 FITC antibody did not work in this experiment. However, data for average CD45 expression in CD34 enriched cultures are discussed in Chapter 3, Figure 3.8.

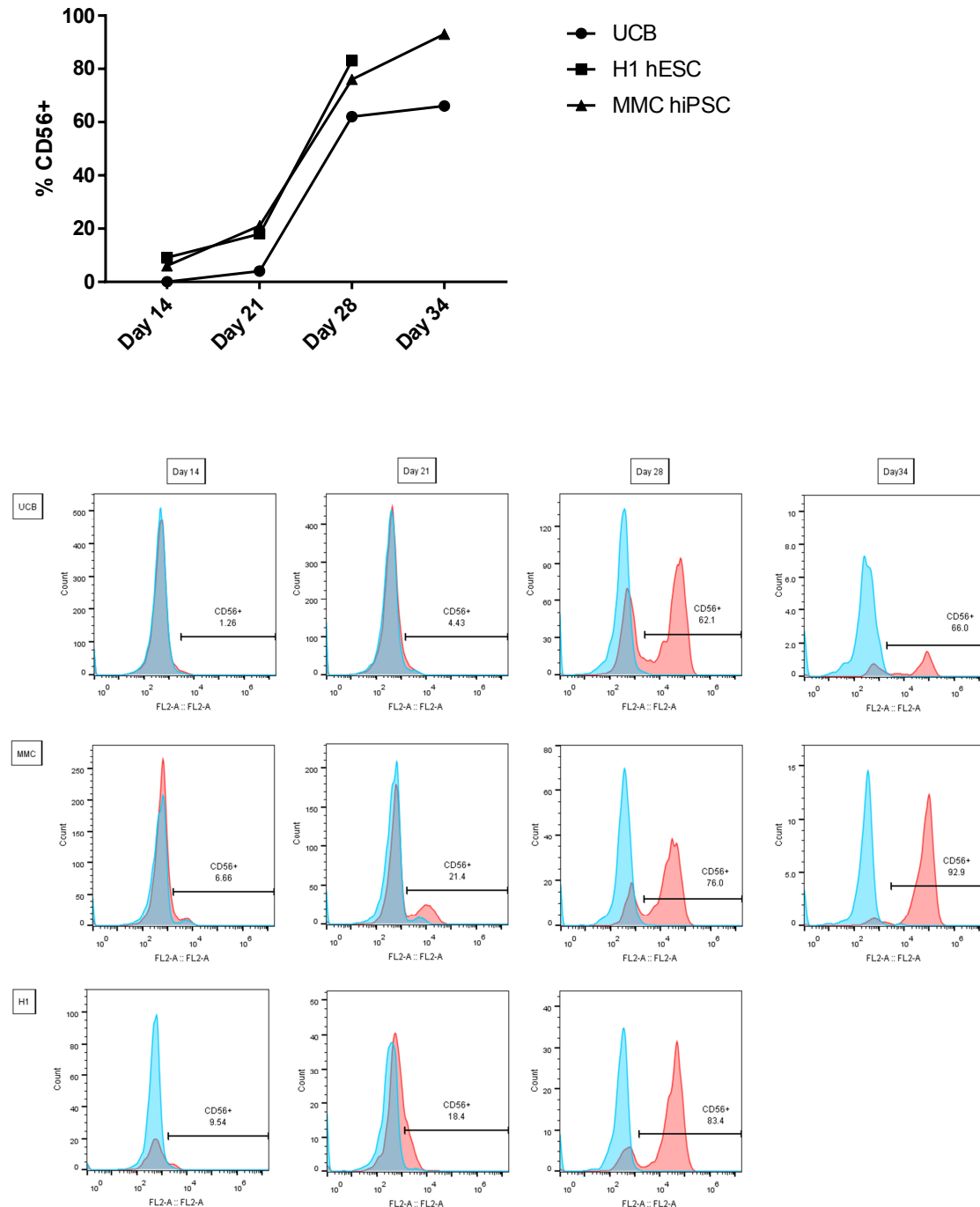


**Figure 4.7 CD34 cell purities for EL08.1D2 NK cell differentiation.** 95% UCB-CD34<sup>+</sup>, 92% H1 hESC-CD34<sup>+</sup> and 68% MMC hiPSC-CD34<sup>+</sup> cell purities. hPSC-CD34<sup>+</sup> cells were derived by OP9 co-culture and all cells enriched by CD34 MACS technology. CD45 FITC antibody did not give a representative staining in this experiment. All gates were set based on corresponding isotype controls.

#### 4.2.4 CD56 is upregulated during differentiation

We monitored the upregulation of CD56 expression by flow cytometry during NK cell generation weekly until day 34 of culture (Figure 4.8). On day 14 of NK cell differentiation, upregulation of 6% and 9% of CD56 can be seen in MMC hiPSC- and H1 hESC-derived cells, respectively. In UCB-derived cells, upregulation of 4% can only be determined a week later on day 21 of culture. At this time point, MMC hiPSC- and H1 hESC-derived cultures express 21% and 18% CD56, respectively. MMC hiPSC-derived cells display a clearly separate peak in the histogram, whereas H1 hESC-derived cells display a slight shift of CD56 expression. Significant upregulation of CD56 occurred in the 4<sup>th</sup> week of differentiation, with 62%, 76% and

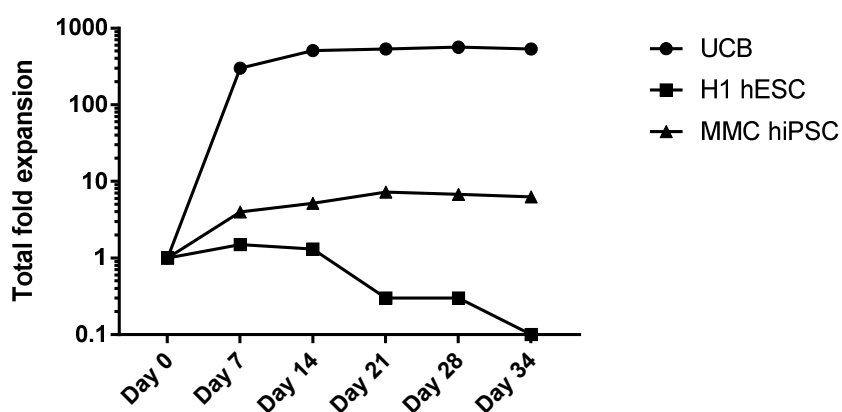
83% of cells expressing CD56 in UCB-, MMC hiPSC- and H1 hESC-derived cell cultures, respectively. On day 34 of EL08.1D2 co-culture, 66% of UCB-derived cells express CD56 and 93% of MMC hiPSC-derived cells. Interestingly, these data suggest, that the upregulation of CD56 occurs earlier and more rapid in hPSCs compared to UCB-derived cells, resulting in higher percentage of CD56 expression on day 34 of NK cell differentiation culture. In the initial study we could observe over 90% of H1-derived cells expressed CD56 whereas JOM hiPSC-derived cells expressed CD56 in only 37% of cells. This could be due to the genetic background of the hiPS cell line. Unfortunately, due to contamination issues, the co-culture with H1 hESC cells had to be discarded before day 34 analysis. UCB-derived cells were affected by contamination after 5 weeks in culture and MMC hiPSC-derived cells were collected for further analysis.



**Figure 4.8 CD56 upregulation during EL08.1D2 NK cell differentiation.** (A) Percentage of CD56 expressing cells during differentiation, based on flow cytometry analysis of total cells. (B) Flow cytometry data show upregulation of CD56 in UCB-, MMC hiPSC- and H1 hESC-derived cells on days 14, 21, 28 and 34.

#### 4.2.5 Cell numbers and expansion during NK cell differentiation

Highly efficient NK cell generation from UCB-derived CD34<sup>+</sup> cells is reported with the EL08.1D2 co-culture system with up to 1x10<sup>6</sup> NK cells generated from 500 CD34<sup>+</sup> cells (Grzywacz, Kataria et al. 2006; Luevano, Domogala et al. 2014). We aimed to determine the expansion potential of hPSC-derived cells under these conditions. Total cells in culture were counted weekly and total fold expansion determined (Figure 4.9). In our culture conditions, UCB-derived cells expanded over 100 fold within the first week and a 700 fold expansion could be observed at day 35 of EL08.1D2 co-culture. The cell expansion of hPSC-derived cells was extremely low to what was observed in UCB-derived cell cultures with less than 10 fold expansion.

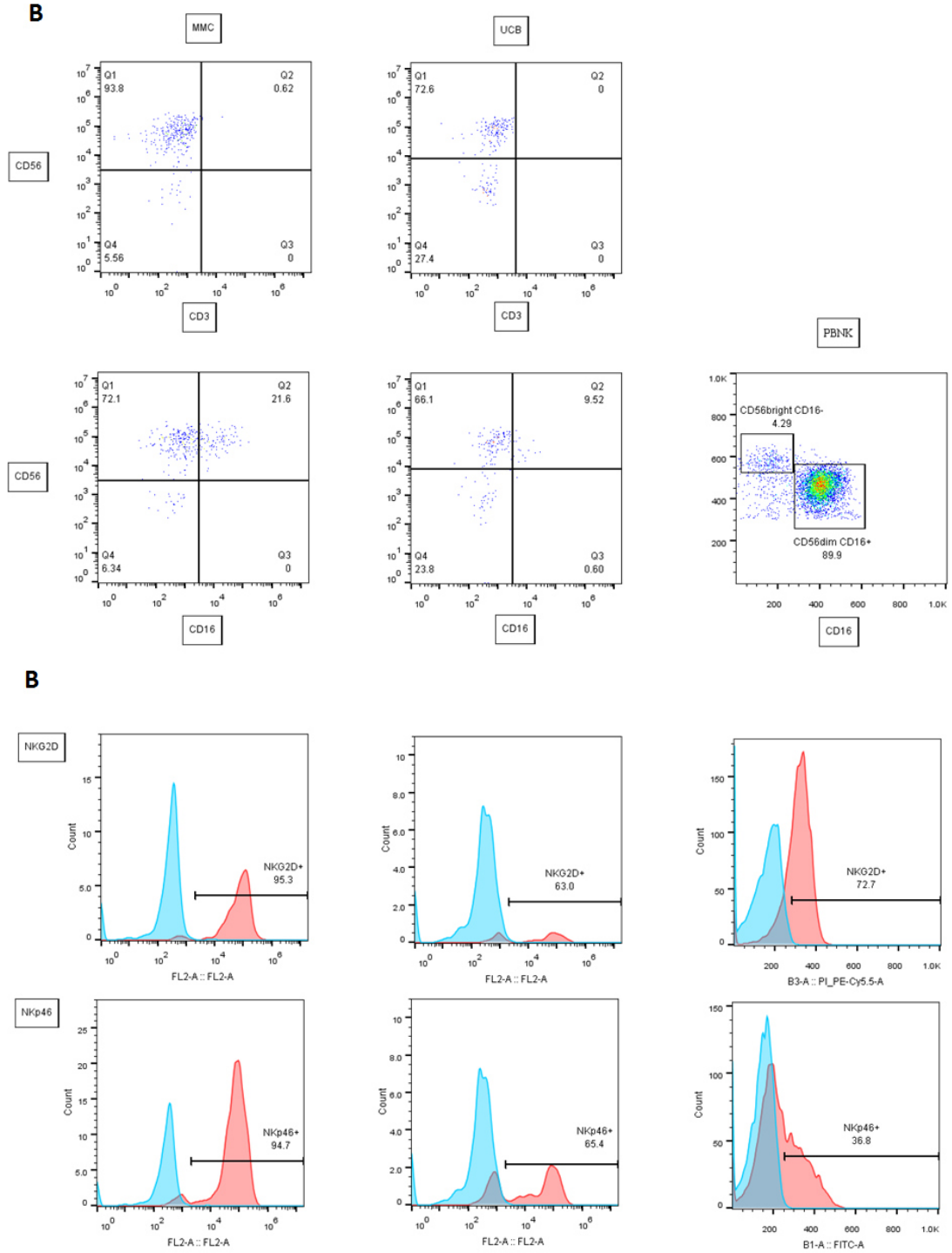


**Figure 4.9 Total fold expansion of cells during EL08.1D2 NK cell differentiation.** UCB-, H1 hESC and MMC hiPSC-derived cell count was determined by trypan blue count. More than 100 fold lower expansion of hPSC-derived cells could be observed compared to UCB-derived cells.

#### 4.2.6 NK cell specific markers are expressed in hPSC-derived cells

To determine if the generated CD56<sup>+</sup> cells expressed NK cell-specific surface markers, we analysed UCB- and MMC hiPSC-derived cells on day 34 by flow cytometry. Importantly, data showed that cells did not express CD3, excluding the presence of T and NKT cells in the culture (Figure 4.10). Sub-populations CD56<sup>dim</sup> and CD56<sup>bright</sup> cannot be distinguished in either UCB- or MMC hiPSC-derived NK-like cells.

Interestingly, CD16 expression was higher on MMC hiPSC-derived cells with 21% CD56<sup>+</sup>CD16<sup>+</sup> cells, compared to 9% CD56<sup>+</sup>CD16<sup>+</sup> cells from UCB-derived cells. Compared to NK cells isolated from peripheral blood, where 90% of cells belong to the cytotoxic CD56<sup>dim</sup>CD16<sup>+</sup> sub-population. Cells derived from both sources – MMC hiPSC and UCB – expressed the activating NK cell marker NKG2D with 95% and 63%, respectively. Furthermore, NKp46 expression could be observed in 95% and 65% of cells in MMC hiPSC- and UCB-derived cells, respectively. Interestingly, these data show higher expression of both NK cell specific markers – NKG2D and NKp46 – in MMC hiPSC-derived cells compared to expression levels observed in UCB-derived NK cells and PBNKs.

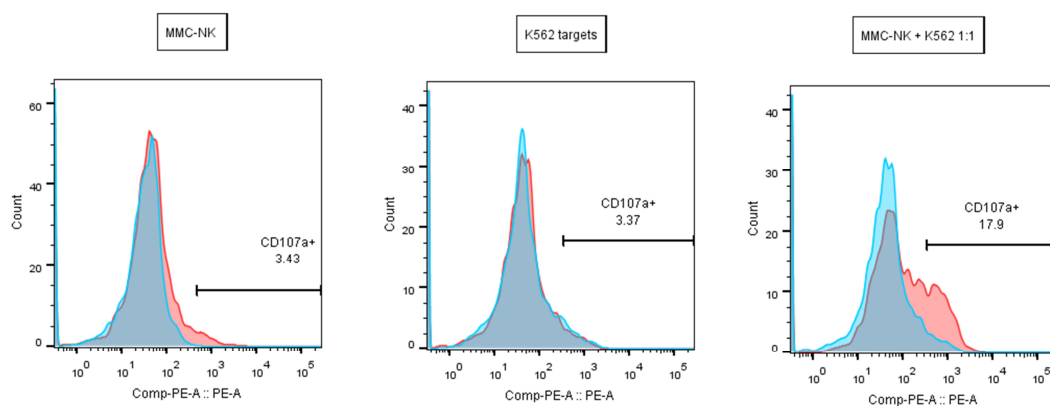


**Figure 4.10 UCB- and hiPSC-derived NK-like cells on day 34 of NK cell differentiation compared to freshly isolated PBNKs. (A) Both MMC hiPSC- and UCB-derived cell display NK cell characteristic expression CD56<sup>+</sup>CD3<sup>-</sup>, with no CD56<sup>-</sup>CD3<sup>+</sup> T cells or CD56<sup>+</sup>CD3<sup>+</sup> NKT cells present in the cultures. 21% and 9% of**

MMC hiPSC- and UCB-derived cells expressed CD16, respectively. (B) NK cell specific markers are expressed in MMC hiPSC-derived cells to 95% NKG2D and 95% NKp46 and UCB-derived cells express 63% NKG2D and 65% NKp46.

#### 4.2.7 MMC hiPSC-derived NK-like cells exhibit cytotoxic function against the leukemic cell line K562 in CD107a degranulation assay

To assess the functional activity of MMC hiPSC-derived NK-like cells, we performed an *in vitro* CD107a degranulation assay. NK-like cells were co-cultured 1:1 with K562 target cells for 2 hours and the degranulation was assessed by the expression of CD107a on the cell surface using flow cytometry. The mean degranulation of MMC hiPSC-derived NK-like cells was  $14 \pm 3.2\%$ ,  $n=3$  (Figure 4.11).



**Figure 4.11 CD107a degranulation assay MMC-derived NK like cells day 35.**

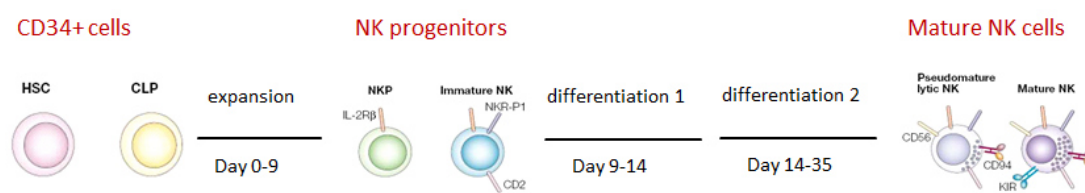
Controls confirm that neither MMC hiPSC-NK like cells nor K562 target cells express CD107a. CD107a is upregulated after co-culture to  $14 \pm 3.2\%$ ,  $n=3$ , indicating cytotoxic efficiency of hiPSC-derived NK like cells.



#### 4.2.8 Serum- and feeder free NK cell differentiation with GBGM medium

To explore the NK cell differentiation potential in serum- and feeder conditions, we used the Glycostem basal growth medium (GBGM) supplemented with a high dose of SCF, IL-7, TPO and Flt3L and a low dose of G-CSF, GM-CSF and IL-6. Differentiation was induced by replacing TPO with IL-15 on day 10 and Flt3L with IL-2 on day 14 (Figure 4.12). This method for NK cell differentiation from freshly isolated UCB-CD34<sup>+</sup> cells is GMP compliant. The initial study reported >15000 fold expansion of the *ex vivo* generated cell product, containing nearly 100% functional NK cells (Spanholtz, Preijers et al. 2011). With gene expression profiling, real-time PCR, flow cytometry and functional analysis, this group characterised UCB-derived NK cells and compared data to PBNKs (dim and bright) (Lehmann, Spanholtz et al. 2012). At day 28, the CD56<sup>+</sup> NK cell population within the culture reached 50% and over 95% at day 35. With gene expression analysis this study shows that relevant NK cell receptors and intracellular proteins for NK cell function are expressed in UCB-derived NK cells, eg. CD56, CD94, CD16 and cytolytic proteins (granzyme A, granzyme B, perforin) (Lehmann, Spanholtz et al. 2012).

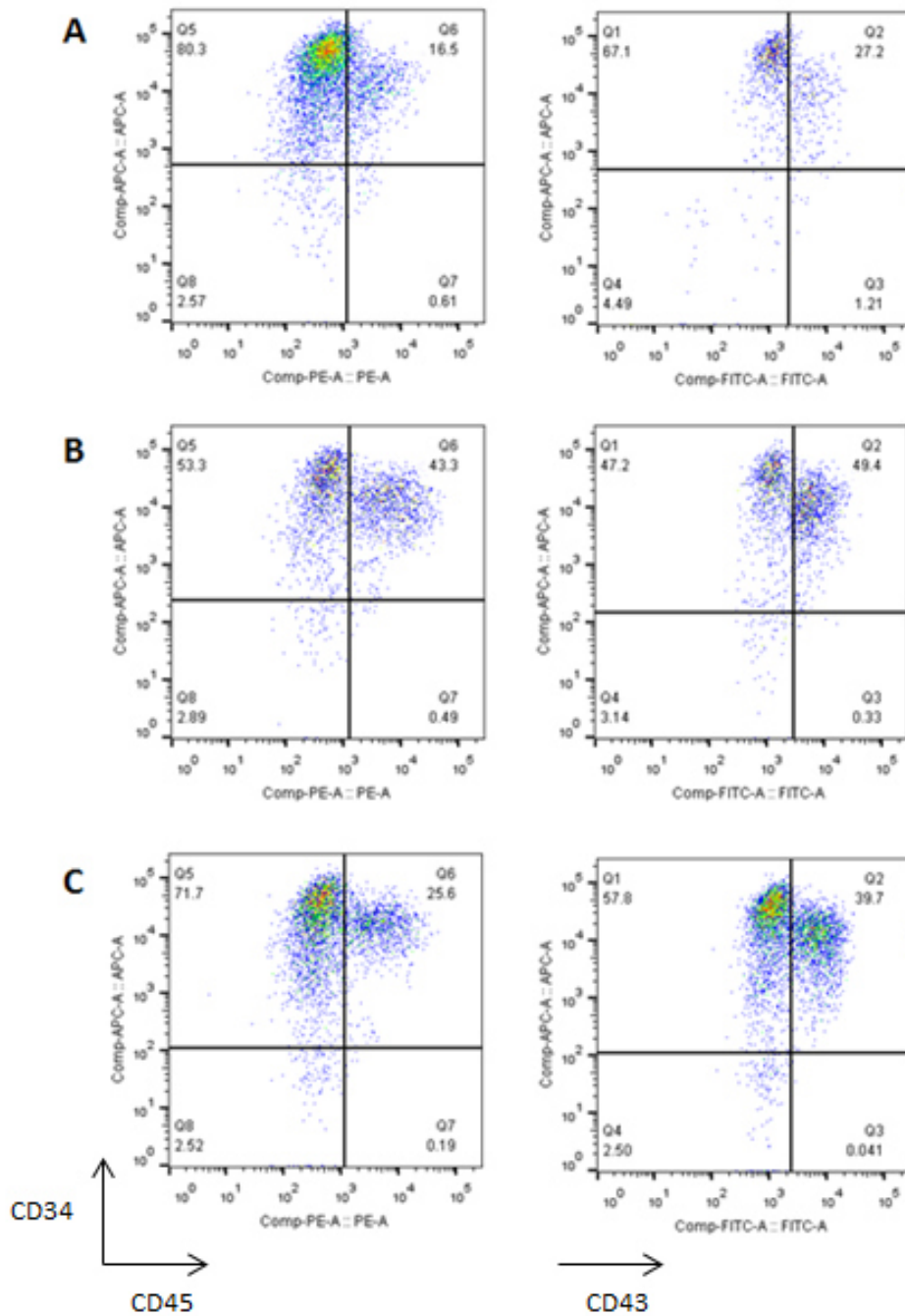
Interestingly, the data suggest that UCB-derived NK cells, differentiated with this method cannot, be classified as conventional CD56<sup>bright</sup> or CD56<sup>dim</sup> sub-populations as seen in PBNKs. Although UCB-derived NK cells display a phenotype similar to CD56<sup>bright</sup> PBNKs, they possess potent cytotoxicity and produce IFN- $\gamma$  upon activation. These data demonstrate, that the *ex vivo* generation of NK cells (from UCB CD34<sup>+</sup> cells) is a promising approach to generate large numbers of cytotoxic lymphocytes for immunotherapy (Lehmann, Spanholtz et al. 2012). HPSC have not been used to generate NK cells with this feeder- and serum free method before. We aimed to derive NK like cells from hPSCs with comparable cell expansion and phenotype as observed in UCB- derived and PBNKs.



**Figure 4.12 Schematic overview of serum- and feeder free NK cell differentiation with GBGM medium.** CD34<sup>+</sup> cells are cultured in a combination of expansion and differentiation media – supplemented with respective cytokines – to generate NK cells within 35 days.

#### 4.2.9 Immunophenotypic characterisation of UCB- and hPSC-derived CD34<sup>+</sup> cells as starting cell population for NK cell differentiation with GBGM medium

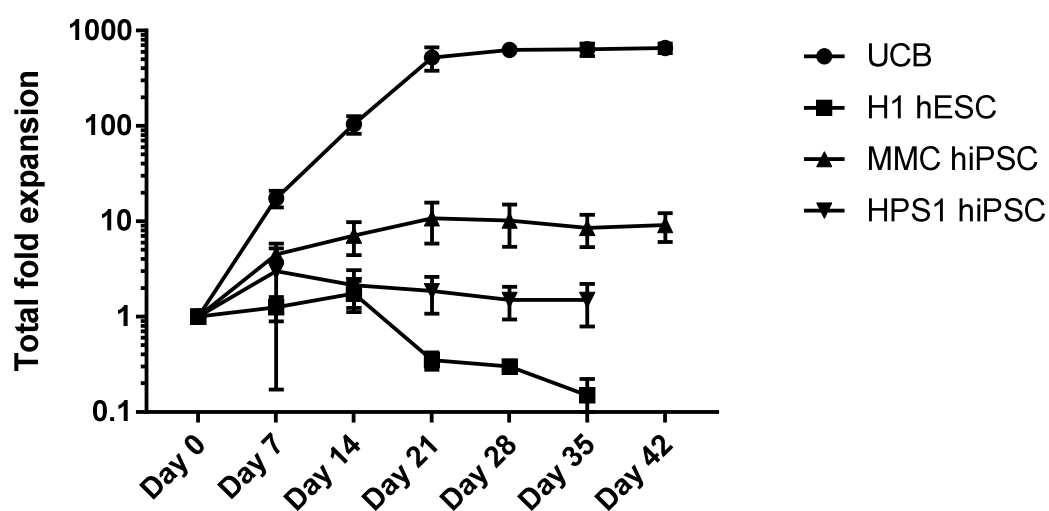
We aimed to establish the serum- and feeder free NK cell differentiation protocol with GBGM in our laboratory, comparing the NK cell generation potential of UCB-, H1 hESC, HPS1 hiPSC- and MMC hiPSC-derived CD34<sup>+</sup> cells. hPSC-derived CD34<sup>+</sup> cells were generated with OP9 co-culture and CD34<sup>+</sup> cells of all three cell sources were enriched with CD34 MACS technology. The purities of CD34 cultures used in this experiment were 96% for H1 hESC-, HPS1 hiPSC- and MMC hiPSC-derived cells (Figure 4.13). Highest CD45 and CD43 co-expression was observed in HPS1 hiPSC-derived CD34<sup>+</sup> cells with 43% and 49%, respectively. 16% of H1 hESC-derived cells co-expressed CD45 and 27% CD43. Co-expression of 25% CD45 and 40% CD43 could be detected in MMC hiPSC-derived cells.



**Figure 4.13 CD34 cell purities for GBGM NK cell differentiation.** CD34<sup>+</sup> MACS enriched cells derived from (A) H1 hESC displayed a purity of 96% CD34 with 16% of cells CD34<sup>+</sup>CD45<sup>+</sup> and 27% CD34<sup>+</sup>CD43<sup>+</sup>. (B) 96% of HPS1 hiPSC-derived cells expressed CD34 with 43% CD34<sup>+</sup>CD45<sup>+</sup> and 49% CD34<sup>+</sup>CD43<sup>+</sup>. (C) Enriched cells derived from MMC hiPSCs expressed 96% CD34 with 25% CD34<sup>+</sup>CD45<sup>+</sup> and 40% CD34<sup>+</sup>CD43<sup>+</sup>. All gates were set based on corresponding isotype controls.

#### 4.2.10 Cell numbers and expansion during NK cell differentiation

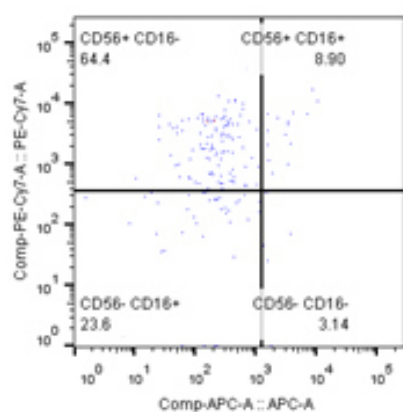
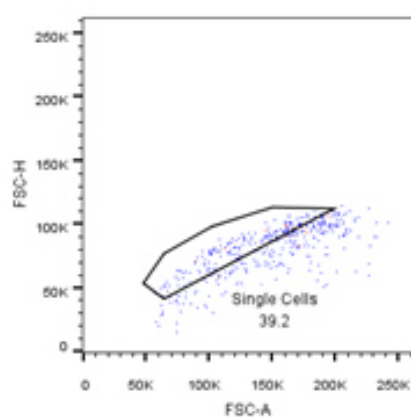
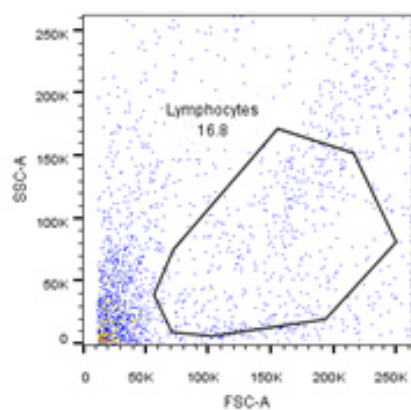
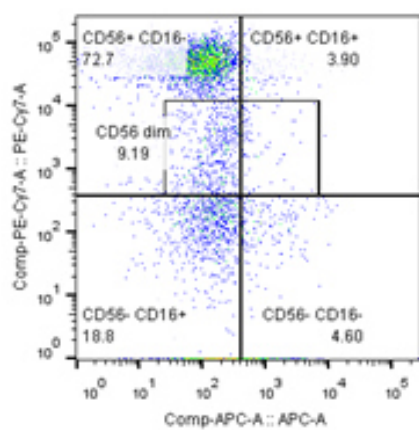
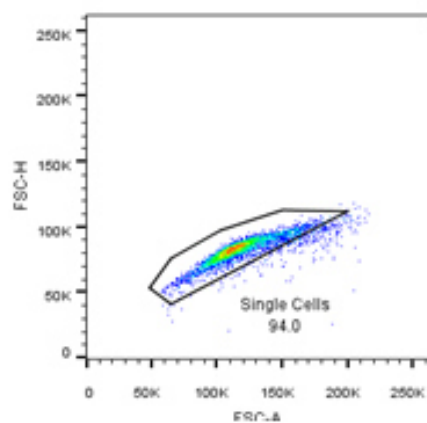
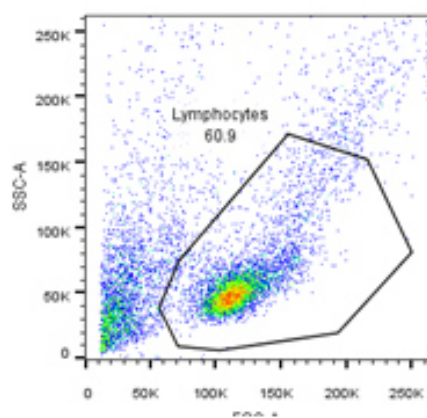
Highly efficient NK cell generation from UCB-derived CD34<sup>+</sup> cells is reported with the GBGM culture system (Spanholtz, Tordoir et al. 2010; Spanholtz, Preijers et al. 2011; Lehmann, Spanholtz et al. 2012). We aimed to determine the expansion potential of hPSC-derived cells under these conditions. Total cells in culture were counted weekly and total fold expansion was determined (Figure 4.14). In our culture conditions, UCB-derived cells expanded 800 fold with highest expansion rate within the first three weeks of culture. The cell expansion of hPSC-derived cells was very low and comparable what could be observed in EL08.1D2 co-culture. The low cell survival and expansion potential of hPSC-derived cells made experimental planning and analysis very challenging and we aimed to keep as many cells as possible in culture until the last stages for characterisation.



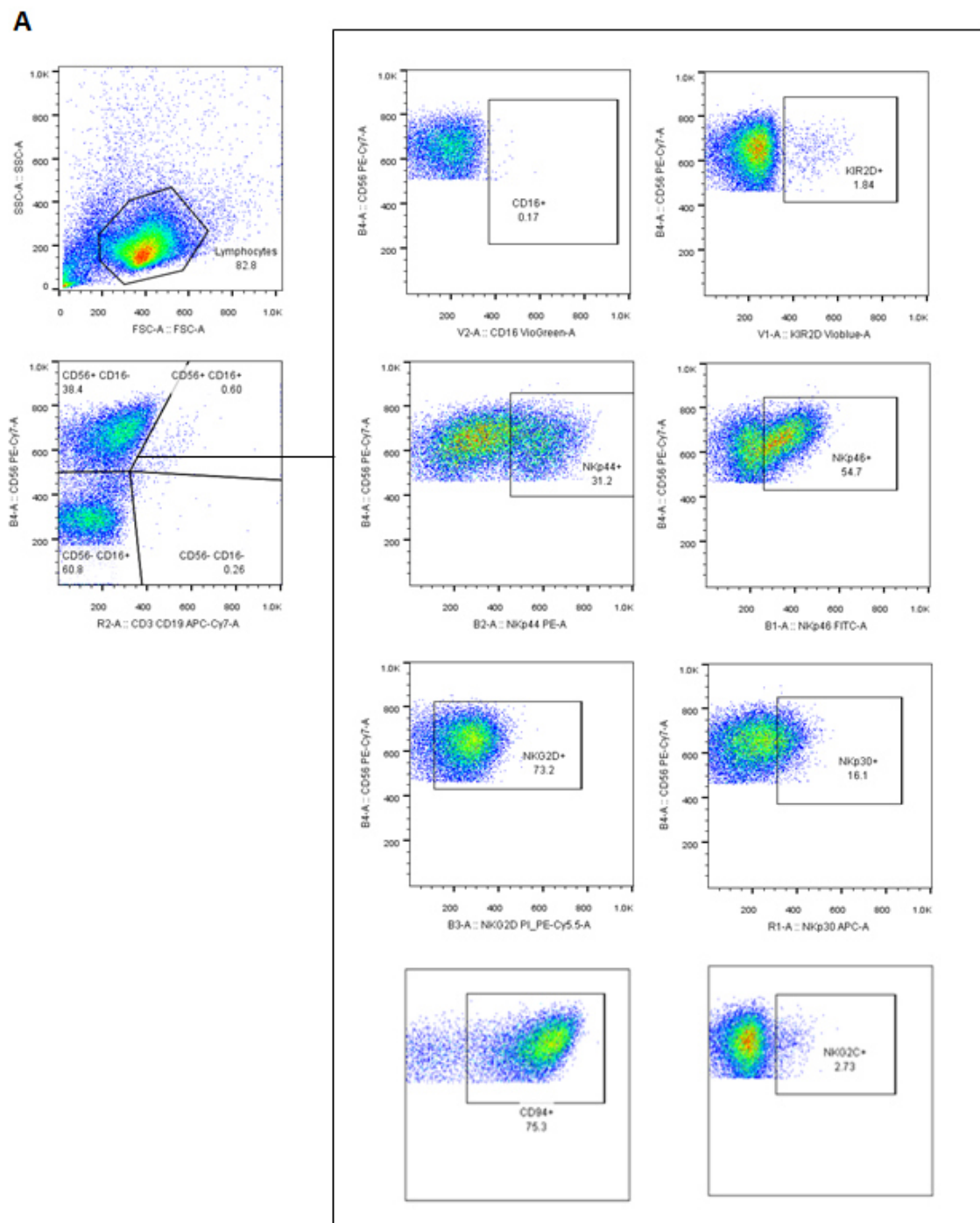
**Figure 4.14 Total fold expansion of cells during EL08.1D2 NK cell differentiation.** UCB-, H1 hESC-, HPS1 hiPSC- and MMC hiPSC-derived cell count was determined by trypan blue count. 100 fold lower expansions of hPSC-derived

cells could be observed compared to UCB-derived cells. Results are presented as Mean $\pm$ SD, n=2.

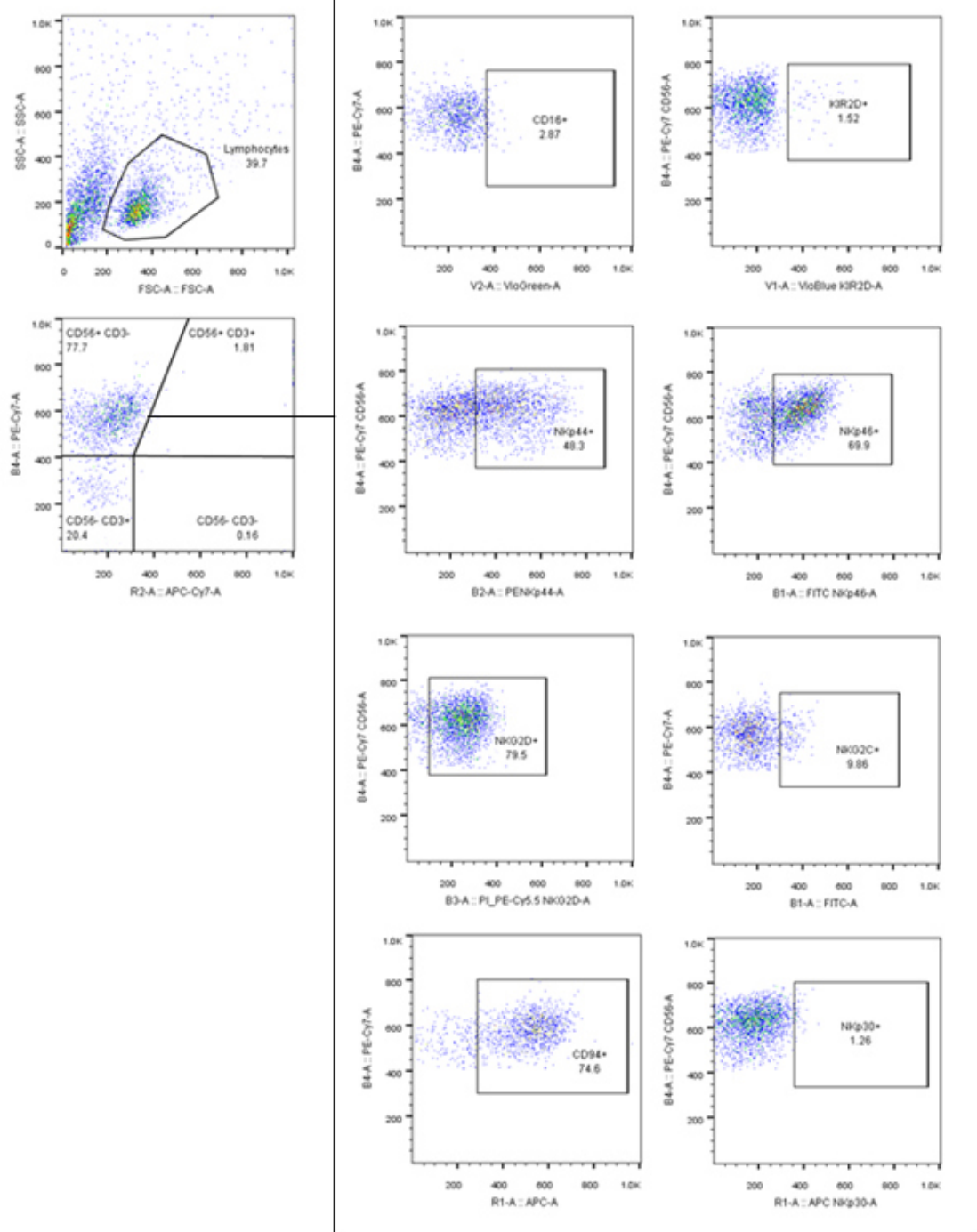
On day 28 and day 35 of NK cell differentiation, we collected cells and performed analysis of surface marker expression by flow cytometry. In HPS1 hiPSC-derived cell cultures we could not observe a distinct cell population in the FSC/SSC profile (Figure 4.15 A). These data suggest that cell survival was poor. Despite the lack of a discrete lymphocyte population, we aimed to gate on the lymphocyte population and could observe cell populations expressing 64% CD56 and 9% CD56<sup>+</sup>CD16<sup>+</sup>. On day 35 of GBGM culture, only cell debris could be observed in HPS1 hiPSC- and H1 hESC-derived cells and cultures were lost for further analysis (Figure 4.15 C). In contrast to HPS1 hiPSC-, MMC hiPSC-derived cells revealed a distinct lymphocyte population based on FSC/SSC profiling and interestingly, cells expressed the NK cell marker CD56 to 95% on day 28 of differentiation (Figure 4.15 B) compared to 38% observed in UCB-derived NK cell cultures (Figure 4.16). Cells generated from UCB-derived CD34<sup>+</sup> cells on day 28 and 35 expressed CD56 in 38% and 78%, respectively (Figure 4.16 A and B). NK cell specific marker expression could be observed to similar levels in week 4 and 5.

**A****B**

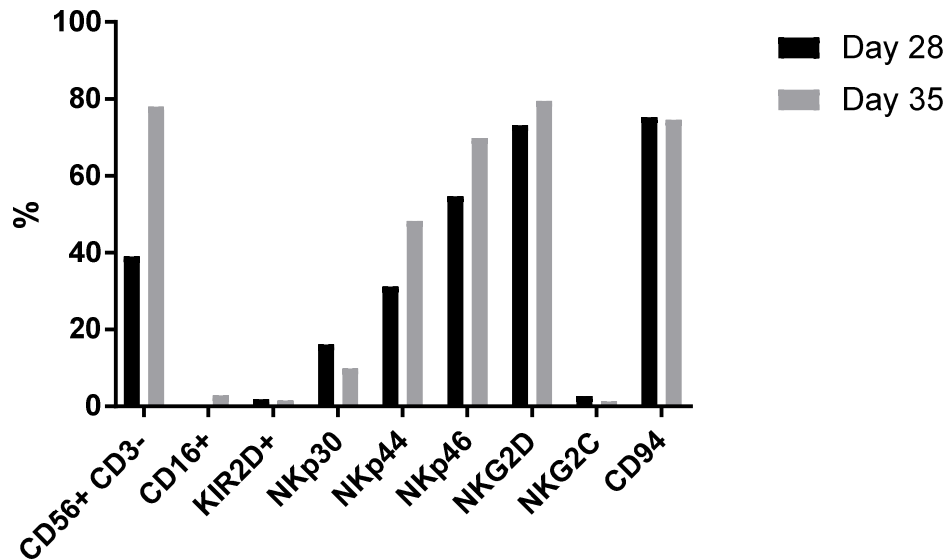
**Figure 4.15** In vitro generated NK cells from hPSC-derived CD34<sup>+</sup> cells on day 28 and 35 of feeder- and serum free GBGM differentiation method. Cells were analysed by flow cytometry using antibodies for NK cell specific surface markers. (A) HPS1 hiPSC-derived cells on day 28, (B) MMC hiPSC-derived cells on day 28.



**B**







**Figure 4.16** *In vitro* generated NK cells from UCB-derived CD34<sup>+</sup> cells on day 28 and 35 of feeder- and serum free GBGM differentiation method. Cells were analysed by multi-colour flow cytometry using a panel of antibodies for NK cell specific surface markers. (A) UCB-derived NK cells on day 28 and (B) day 35 of GBGM culture. Cells for surface marker analysis were gated on the CD56<sup>+</sup>CD3<sup>-</sup> cell population. (C) Expression data in % are summarised in a bar chart, n=1.

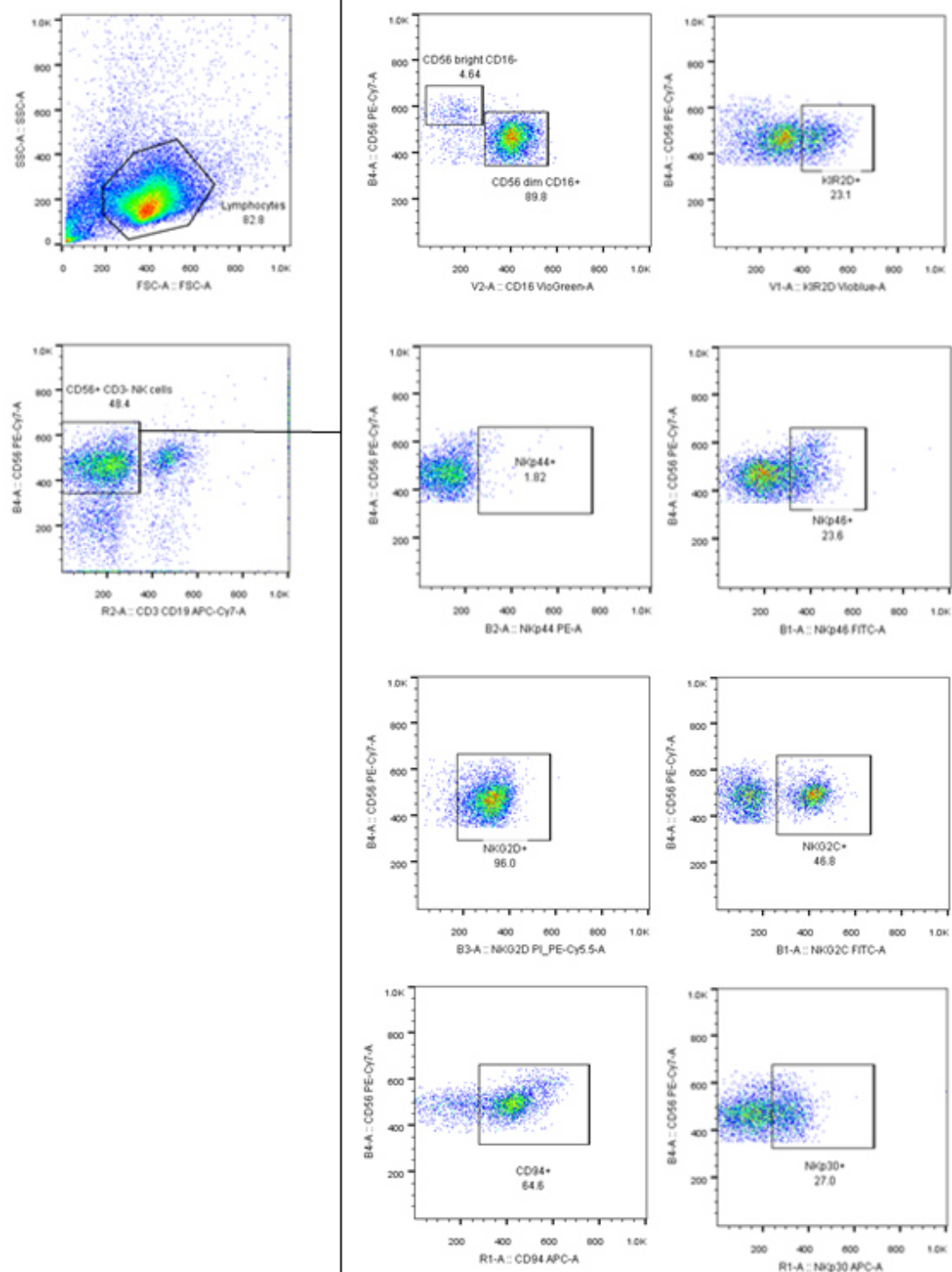
#### 4.2.11 HiPSC-derived NK like cells display surface marker expression close to UCB-derived NK cells and freshly isolated PBNKs

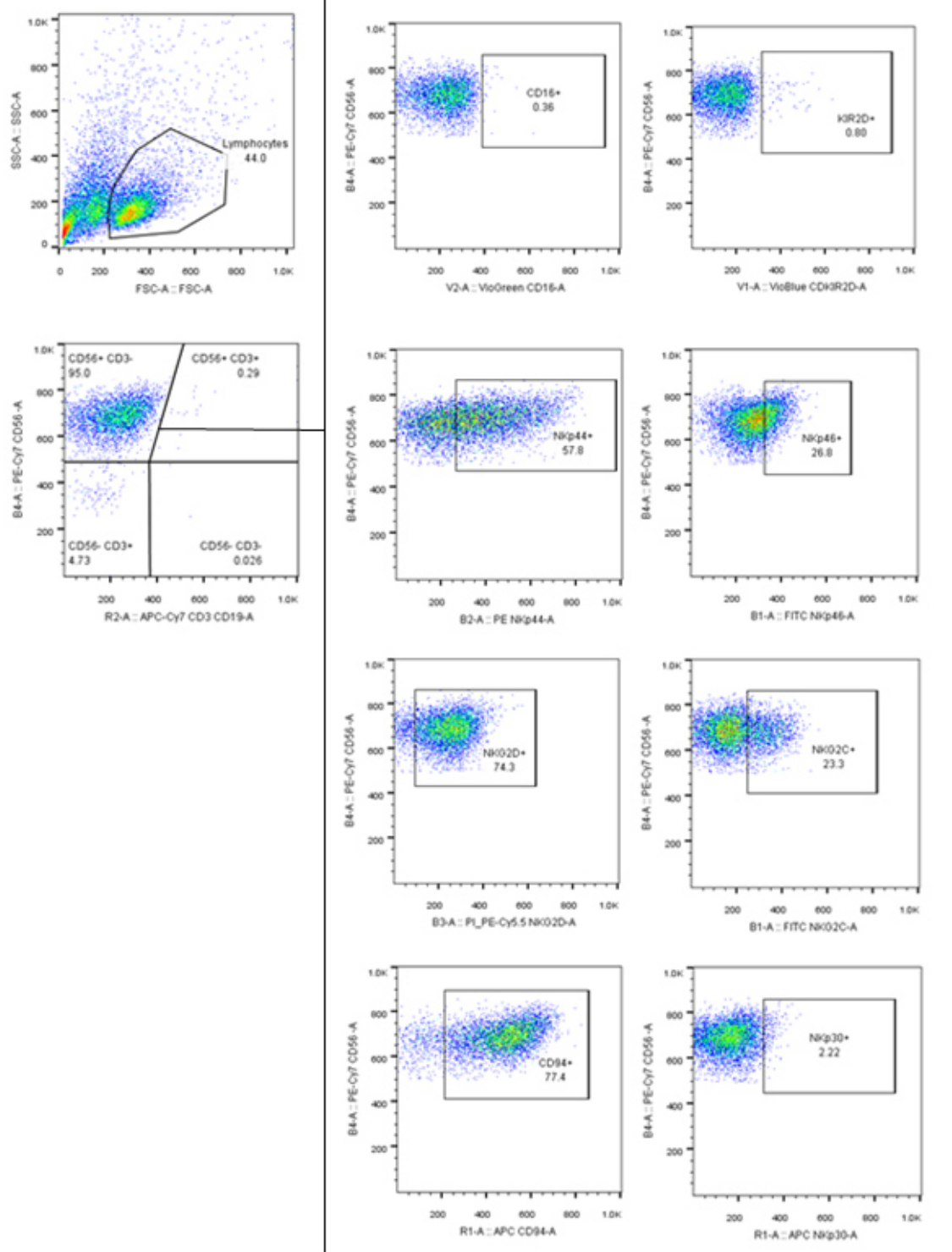
To investigate if hiPSC-derived NK like cells express NK cell specific surface makers, to a similar level compared to UCB-derived NK cells and freshly isolated PBNKs, we analysed GBGM-derived cells on day 42 by multi-colour flow cytometry using a panel of antibodies for NK cell specific surface markers. PBMCs were isolated from a healthy donor by density gradient and NK cells were enriched by MACS NK cell separation. Cells were gated on the CD56<sup>+</sup>CD3<sup>-</sup> NK cell population (Figure 4.17). PBNKs displayed the two main subpopulations CD56<sup>bright</sup>CD16<sup>-</sup> and

CD56<sup>dim</sup>CD16<sup>+</sup> (Figure 4.17 A). Analysis of UCB- and hiPSC-derived NK like cells revealed that derived cells cannot be classified as conventional CD56<sup>bright</sup> or CD56<sup>dim</sup> sub-populations as seen in PBNKs (Figure 4.17 B, C). CD16 expression could not be observed in UCB-derived and hiPSC-derived NK like cells. However, 5% of hiPSC-derived NK like cells expressed panKIR2D on their surface, higher compared to 0.8% observed in UCB-derived NK-like cells. Interestingly, we observed higher panKIR2D expression on day 28 and day 35 of GBGM differentiation in UCB-derived cells (Figure 4.16). CD94 is expressed by 84% of MMC hiPSC-derived NK like cells, compared to 77% of UCB-derived NK cells and 64% PBNKs. NCRs NKp44 is expressed only on 2% of fresh PBNKs, to 58% UCB-derived and 93% MMC hiPSC-derived NK like cells. NKp30 is expressed in 27% of PBNKs, 2% in UCB-derived and 94% hiPSC-derived NK like cells. NKG2D is expressed in 96% PBNKs, 74% UCB- and 77% hiPSC-derived NK like cells. NKp46 and NKG2C could not be analysed in hiPSC-derived cells because FACS panel tube 1 and 2 were combined to only 1 tube.

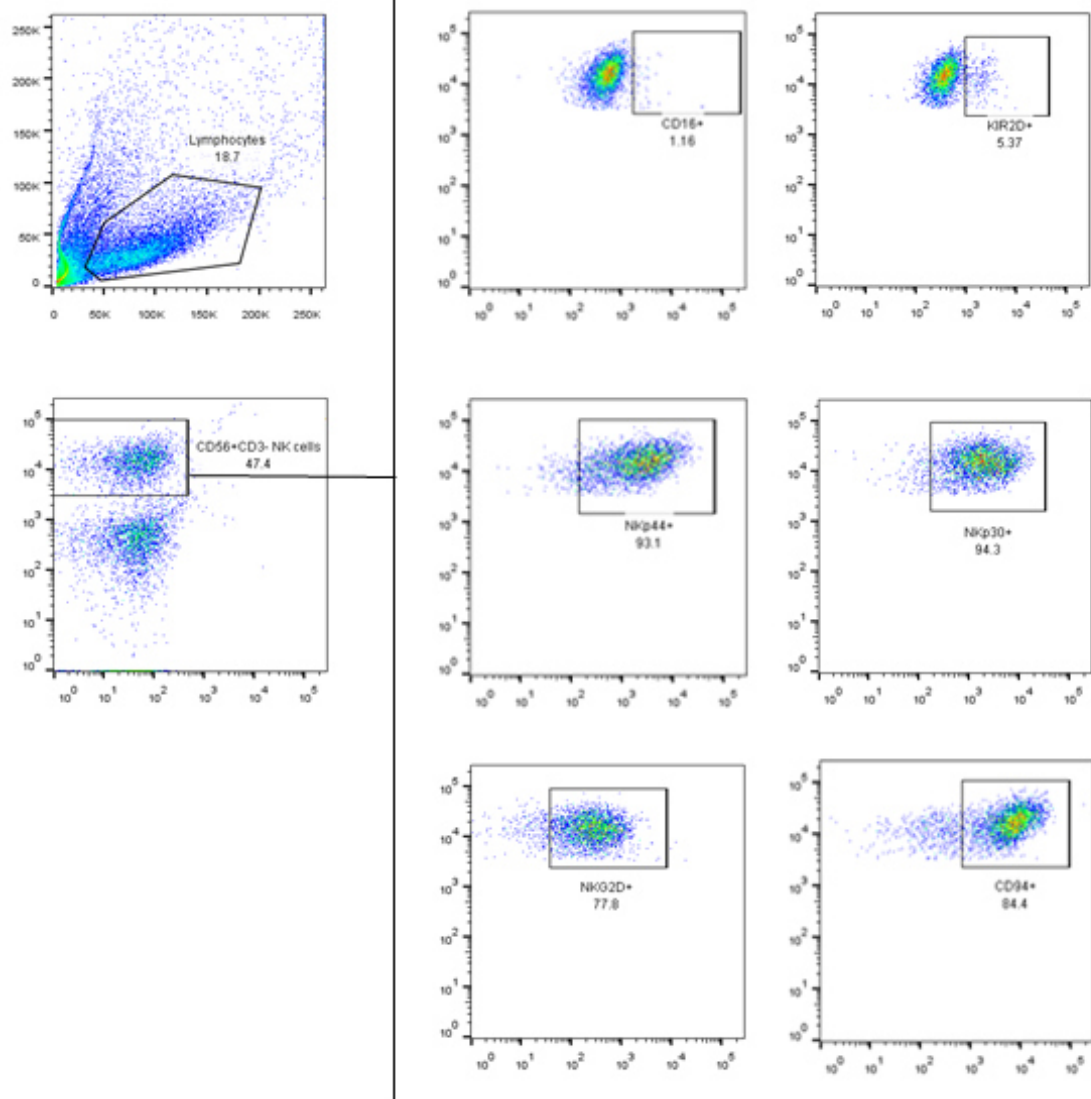
Surface expression of NKG2A and NKG2C should be linked with CD94 expression as NKG2 chains can only be transported to the cell surface with CD94 (Lopez-Botet, Bellon et al. 2000). NKG2C is donor dependent in PBNKs (Guma, Cabrera et al. 2006).

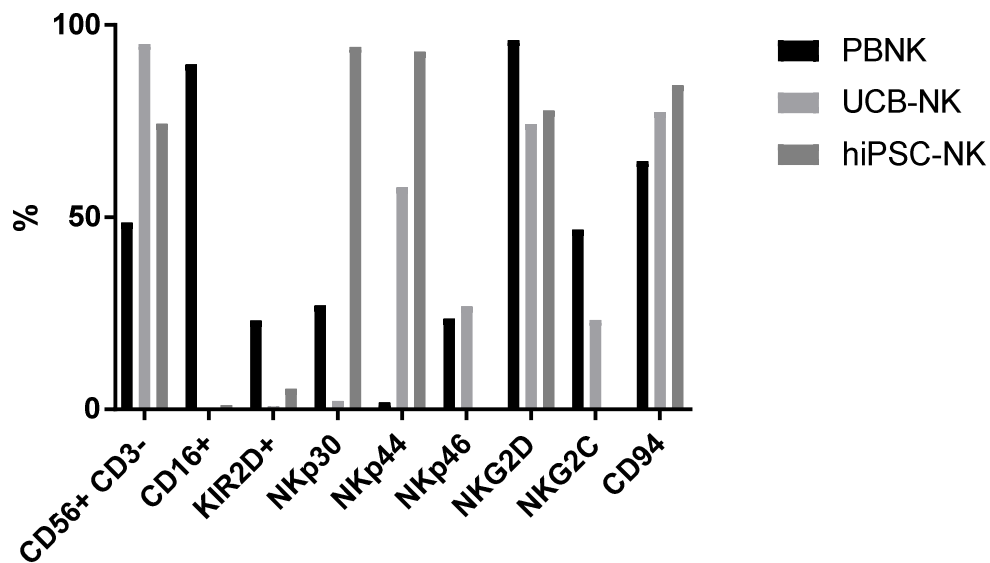
**A**



**B**

**C**





**Figure 4.17 *In vitro* generated NK like cells on day 42 of feeder- and serum free GBGM differentiation method.** Cells were analysed in parallel by multi-colour flow cytometry using a panel of antibodies for NK cell specific surface markers. (A) Primary NK cells isolated from fresh blood, (B) UCB-derived NK cells day 42 and (C) MMC hiPSC-derived NK like cells day 42 of GBGM culture. Cells for surface marker analysis were gated on the CD56<sup>+</sup>CD3<sup>-</sup> cell population. (C) Expression data in % are summarised in a bar chart, n=1.

### 4.3 Discussion

In this chapter we investigated if hiPSC-derived CD34<sup>+</sup> cells (discussed in chapter 3) have the potential to differentiate to cells with NK cell specific surface marker expression and killing capacity. We adapted two different *in vitro* derivation protocols for the generation of NK like cells from hPSC – the co-culture on murine fetal liver cells and a feeder-free culture with GBGM medium. The co-culture on feeder cells has been described in the literature for NK cell generation from UCB-derived, hESC- and hiPSC-derived CD34<sup>+</sup> cells (Knorr, Ni et al. 2013) (Grzywacz, Kataria et al. 2006) (Luevano, Domogala et al. 2014) (Woll, Martin et al. 2005). In contrast, the feeder-free *in vitro* derivation with GBGM medium has so far only been described for UCB-derived CD34<sup>+</sup> cells (Lehmann, Spanholtz et al. 2012). We compared the kinetics of differentiation and phenotype of generated NK like cells to those obtained from UCB-derived CD34<sup>+</sup> cells and PBNKs.

The first major observation was that the expansion of hPSC- (both hESC and hiPSC) derived cells was significantly lower than what was observed with UCB-derived CD34<sup>+</sup> cells. The cell expansion was lowest with hESC-derived cells and was slightly increased with hiPSC-derived cells with less than 10 fold expansion in NK cell differentiation medium. In contrast, UCB-derived CD34<sup>+</sup> cells expanded up to 800 fold. This could be observed in both, co-culture with feeder cells and feeder-free GBGM culture conditions. Total cell expansion of UCB-derived CD34<sup>+</sup> cells with EL08.1D2 co-culture was published with 2000 fold (Grzywacz, Kataria et al. 2006). Even higher expansion was reported with GBGM culture conditions with over 15000 fold expansion within five weeks of differentiation (Spanholtz, Tordoir et al. 2010). In contrast, the expansion potential of hESC-derived cells is reported to be significantly lower. Knorr et al. describe 56.8 fold expansion of hESC-derived cells with EL08.1D2 co-culture and 40.4 fold in feeder-free conditions, which is higher than what was observed in our study but still significantly lower than UCB-derived cell

expansion (Knorr, Ni et al. 2013). This study used the H9 hES cell line compared to H1 hESCs used in our study which might exhibit dissimilar differentiation potential. Furthermore, haematopoietic progenitors were derived by spin EB method and EBs were directly transferred to NK cell differentiation conditions without CD34<sup>+</sup> sorting (Knorr, Ni et al. 2013). Interestingly, the expansion potential of hiPSC-derived cells in *in vitro* NK cell differentiation is not discussed in this study. A different article described 2 log expansion of H9 hESC-derived cells after 3-5 weeks of culture (Woll, Martin et al. 2005). This group sorted for CD34<sup>+</sup>CD45<sup>+</sup> progenitor cells derived by M210-B4 co-culture. Sorting for the double positive cell population could be more specific to haematopoietic progenitors which could explain the increased proliferation rate of these cells.

The low expansion potential could be due to various reasons. Firstly, it could be partly because the hPSC-derived CD34<sup>+</sup> cell population is heterogeneous and is partially primed towards non-haematopoietic lineages. As discussed in Chapter 3, our data suggest that the hPSC-derived CD34<sup>+</sup> cells might encompass only a small fraction of cells with haematopoietic or more specifically lymphocyte or NK cell differentiation potential, based on gene expression analysis and colony forming ability as assessed in CFU assays. Secondly, hPSC-derived haematopoietic progenitors could be less mature or primed towards other haematopoietic lineages and they might not express receptors necessary for the induction of NK cell lineage differentiation.

Despite the difficulties observed with cell expansion, our data suggest that CD56<sup>+</sup>CD3<sup>-</sup> NK-like cells can be generated from both, the H1 hES and the MMC patient-derived hiPS cell line (derived from fibroblasts). In EL08.1D2 co-culture, CD56 is upregulated in both, hESC and hiPSC-derived cells, to up to 80% within 28 days of culture. Interestingly, the percentage of CD56 expression is higher in hPSC-derived cells compared to UCB-derived cells, encompassing 93% and 72% of cells



on day 34, respectively. However, interpretation of these results needs to be done with caution because data were only collected from one experiment due to low cell numbers, time constraints and contamination issues. On day 34 of NK cell differentiation, MMC hiPSC-derived NK like cells co-express CD16 slightly higher compared to UCB-derived NK cells and no distinct CD56<sup>bright</sup> and CD56<sup>dim</sup> sub-populations can be distinguished, compared to freshly isolated PBNKs with 90% of NK cells categorized as CD56<sup>dim</sup>CD16<sup>+</sup>. CD56<sup>bright</sup> NK cells might be in an earlier maturation stage and future lines of investigations could test if hiPSC-derived NK cells can further mature to CD56<sup>dim</sup> sub-population. Furthermore, the characterisation in regards to cytokine release and comparison to different ILC groups would be of interest. Interestingly, hiPSC-derived NK like cells express higher NKG2D and NKp46 activation markers compared to UCB-derived NK cells and PBNKs. Our data further suggest that EL08.1D2 hiPSC-derived NK like cells are capable of killing K562 cells in an *in vitro* killing assay. Low or absent CD16 would suggest that hPSC-derived NK cells are not capable of ADCC. It would be interesting to investigate this further and what other effector functions might be triggered upon target cell recognition.

We further investigated if NK like cells can be generated in feeder-free conditions. Generation of hiPSC-derived NK cells under GMP conditions is of interest for clinical application for immunotherapy. Knorr et al. describe feeder-free NK cell generation from hiPSCs (Knorr, Ni et al. 2013). In contrast to this study, we used hiPSCs derived from fibroblasts under GMP compliant, integration-free conditions. Furthermore, the use of GBGM medium – which was shown to efficiently differentiate and highly expand UCB-derived haematopoietic stem cells (Spanholtz, Tordoir et al. 2010) – was not tested for hPSC-derived NK cell differentiation before. Our data suggest that NK like cells can be generated from the MMC hiPSC-derived CD34<sup>+</sup> cells. Consistent with data from EL08.1D2 co-culture, cell expansion was

extremely low during differentiation with over 100 fold less total fold expansion. Interestingly, MMC hiPSCs survived and expanded best with almost 10 fold expansion, compared to HPS1 hiPSCs and H1 hESCs which could not be cultured until the end of the experiment.

After 42 days of NK cell differentiation, we compared the phenotype by flow cytometry of MMC hiPSC-, UCB-derived and peripheral blood NK cells. Based on our data, the MMC hiPSC-derived NK cells generated with GBGM medium cannot be strictly classified as CD56<sup>bright</sup> or CD56<sup>dim</sup> subpopulations on day 42 of culture, compared to populations present in PBNK cells. This goes confirm with what has been reported in the literature on UCB-derived NK cells (Spanholtz, Tordoir et al. 2010) (Lehmann, Spanholtz et al. 2012) and what can be observed in our control cultures. In contrast to what is published in the literature, UCB-derived NK cells did not express CD16 in our experiment.

MMC hiPSC-derived NK like cells express major NK cell specific markers, including KIR2D , CD94, NKp44 and the activating receptor NKG2D to comparable levels with UCB-derived and peripheral blood NK cells. Interestingly, we observed higher KIR2D and NKp30 expression in MMC hiPSC-derived NK like cells compared to UCB-derived cells.

In summary, our data suggest that NK like cells can be generated from hPSCs differentiated with two individual derivation protocols. Derived cells expressed CD56 in the absence of CD3 and the NK cell-specific marker expression is comparable to levels on UCB-derived and peripheral blood NK cells. Furthermore, we show that K562 target cells can be lysed in an *in vitro* degranulation assay.

The main challenges we observed in this study was low cell expansion and contamination issues due to long derivation protocols. Low cell numbers made it extremely difficult to rigorously study phenotype and functionality.

## **Chapter 5 Transcription factor-driven NK cell differentiation with E4bp4**

### **5.1 Introduction**

In my work so far I have utilized the conventional strategy to generate NK cells from hiPSCs via an intermediate differentiation step to CD34<sup>+</sup> haematopoietic progenitor cells. Although this approach results in functional NK cells phenotypically comparable to cord blood-derived NK cells - there are a couple of obstacles: (1) the long derivation period of 7 to 8 weeks and (2) low NK cell numbers. To overcome these problems we explored the alternative approach of transcription factor-driven differentiation. The underlying idea is to identify key transcriptional regulators, specific for the desired cell type (NK cells in my study) and to over-express them in hiPSCs. Our hypothesis is that this will directly drive the differentiation into the lineage of interest and allows bypassing limiting steps in the development. This concept has been described in several studies, for example direct induction of the haemato-endothelial program in hiPSCs (Elcheva, Brok-Volchanskaya et al. 2014). NK cell development and the transcriptional control have been studied well in mouse settings. With knock out models the roles of a number of key transcriptional regulators have been identified and their phenotypes suggest that they act in different time points in NK cell development. Several factors, such as Eomes, Id2, MEF and Tox have been reported to regulate NK cell maturation and act relatively late in NK cell development (Male, Nisoli et al. 2014).

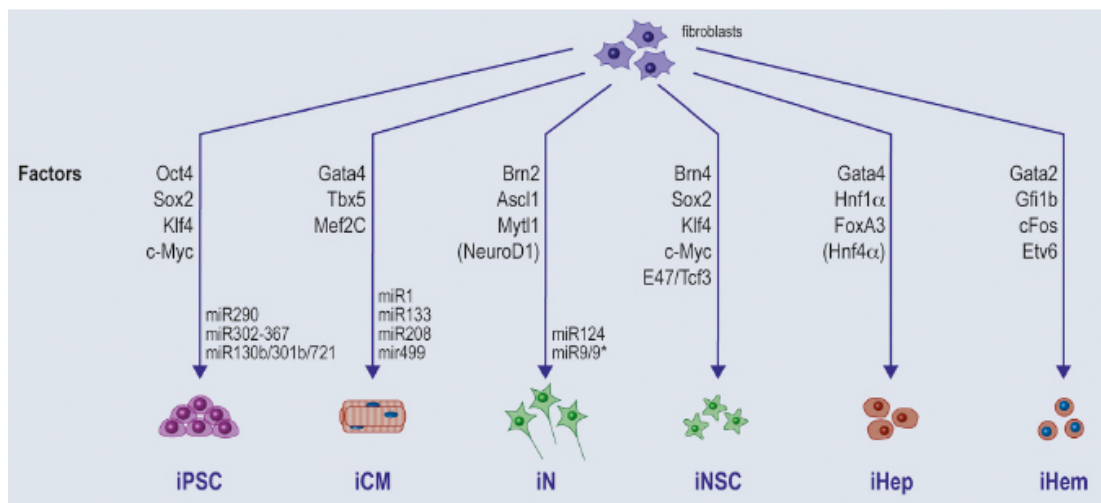
In contrast, the transcription factor E4bp4 (Nfil3) has been shown to act early in NK cell development. Data suggest a possible role as a lineage commitment factor in NK cell development controlling the development of NK cell progenitors (NKPs) from common lymphoid progenitors (CLPs) (Gascoyne, Long et al. 2009; Male,

Nisoli et al. 2014). The research group led by Hugh Brady further showed that in the absence of E4bp4, IL-15-responsive CD122<sup>+</sup> NKPs fail to develop (Male, Nisoli et al. 2014). This suggests that E4bp4 is required for the expression of IL-15 receptor (CD122) and that E4bp4 acts upstream of IL-15 signaling. This has been an interesting observation because IL-15 has previously been considered as the definitive factor required for NK cell differentiation. Furthermore, this study revealed that E4bp4 directly regulates the expression of the transcription factors Eomes and Id2, but not T-bet which can also rescue the E4bp4<sup>-/-</sup> phenotype and might act in a different pathway. Over-expression of E4bp4 in murine CLPs enhances NK cell production *in vitro* (Male, Nisoli et al. 2014). The role of E4bp4 in human NK cell development has not been studied as extensively as in mouse NK cell development.

We aimed to express E4bp4 at different stages in our *in vitro* NK cell differentiation model. Our hypothesis is that the over-expression of human E4bp4 in cord blood and hiPSC-derived CD34<sup>+</sup> cells will increase NK cell numbers with improved cytotoxic effect in NK cell differentiation with GBGM medium. We further hypothesised that the expression of E4bp4 in pluripotent stem cells will directly induce the NK cell developmental program which will allow us to bypass the limiting HSC stage in our *in vitro* culture model.

Furthermore, we aimed to explore the strategy of direct lineage reprogramming (transdifferentiation) for differentiating functional NK cells from hiPSCs. Transdifferentiation describes the reprogramming of one specialised cell type into another by either over-expression of lineage-specific transcription factor or adding small molecules, micro-RNAs or epigenetic regulators. Various cell types in both, mouse and human, have been generated with this approach as summarized in the review of Pfaff et al. (Pfaff and Cantz 2013) (Figure 5.1). We hypothesised, that the

over-expression of an NK cell-specific transcriptional regulator in somatic cells (eg. fibroblasts, endothelial or blood cells) can directly induce the NK cell program. This method will not require the intermediate pluripotent stem cell stage and if feasible it will generate new sources for the derivation of NK cells.

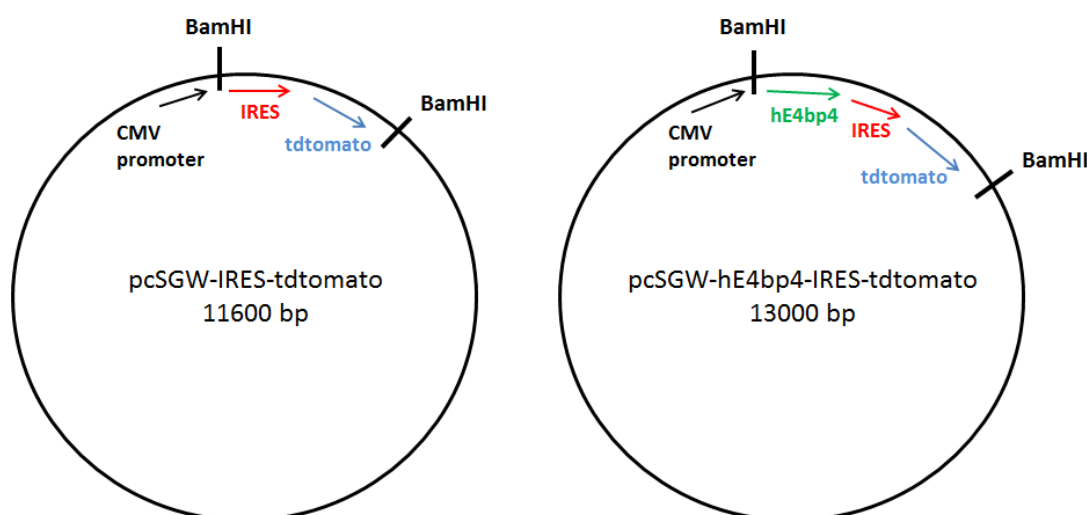


**Figure 5.1 Fibroblast-derived members of the iClub.** (Pfaff and Cantz 2013)

Direct lineage reprogramming of fibroblasts by expression of transcription factors or microRNAs (miRNAs) into iPSC (induced pluripotent stem cells), iCM (induces cardiomyocytes), iN (induced neurons), iNSC (induced neural stem cells), iHep (induced hepatocytes) and iHem (induced haematopoietic cells).

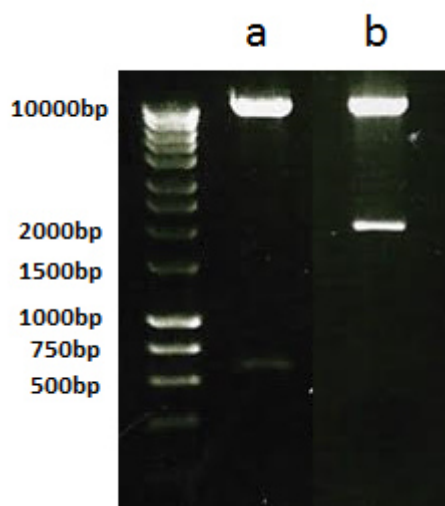
## 5.2 Results

We received the human E4bp4 vector cloned into a lentiviral backbone and the mock control plasmid from a collaborator (Hugh Brady lab): pcSGW-IRES-tdtomato (0.4µg/ml) and pcSGW-hE4bp4-IRES-tdtomato (0.4µg/ml). Sequences for these vectors were not provided, see schematic representation of the plasmids in Figure 5.2. Both plasmids were transfected into E.coli and plasmid DNA purified by mini- and maxi preps.



**Figure 5.2 Schematic plasmid map of original lentivirus over-expression vectors.**

To verify the correct size of the amplified plasmids, a restriction digest with BamHI was performed. The correct fragment sizes were confirmed as followed: backbone 11000bp + 600bp for mock construct (Figure 5.3 a) and 11000bp + 2000bp for E4bp4 plasmid (Figure 5.3 b). The described plasmid preps were used for virus production.



**Figure 5.3 Restriction digest with BamHI.** Correct sizes of plasmids for virus production were confirmed by restriction digest with BamHI. (a) 11000bp + 600bp in mock construct and (b) 11000bp + 2000bp in E4bp4 construct. A 1kb DNA ladder was used in gel electrophoresis.

### 5.2.1 Virus titration

To determine the lentiviral titer of mock (pcSGW-IRES-tdtomato) and E4bp4 (pcSGW-hE4bp4-IRES-tdtomato) lentivirus, we transduced HEK293T cells with dilutions of lentivirus and measured tdtomato expression on day 3 post-transduction (Figure 5.4). 64% of mock transduced cells expressed tdtomato with 1:10 (5µl) and 13% with 1:100 (0.5 µl) dilution. 34% of E4bp4 transduced cells expressed tdtomato with 1:10 (5µl) and 5% with 1:100 (0.5 µl) dilution. Titer was calculated as follows:

Mock

0.5µl of viral supernatant .....13% of  $5 \times 10^4$  HEK293T cells

1000µl of viral supernatant .....13% of  $1 \times 10^8$  HEK293T cells

$$= 1.3 \times 10^7 \text{ infectious units/ml}$$

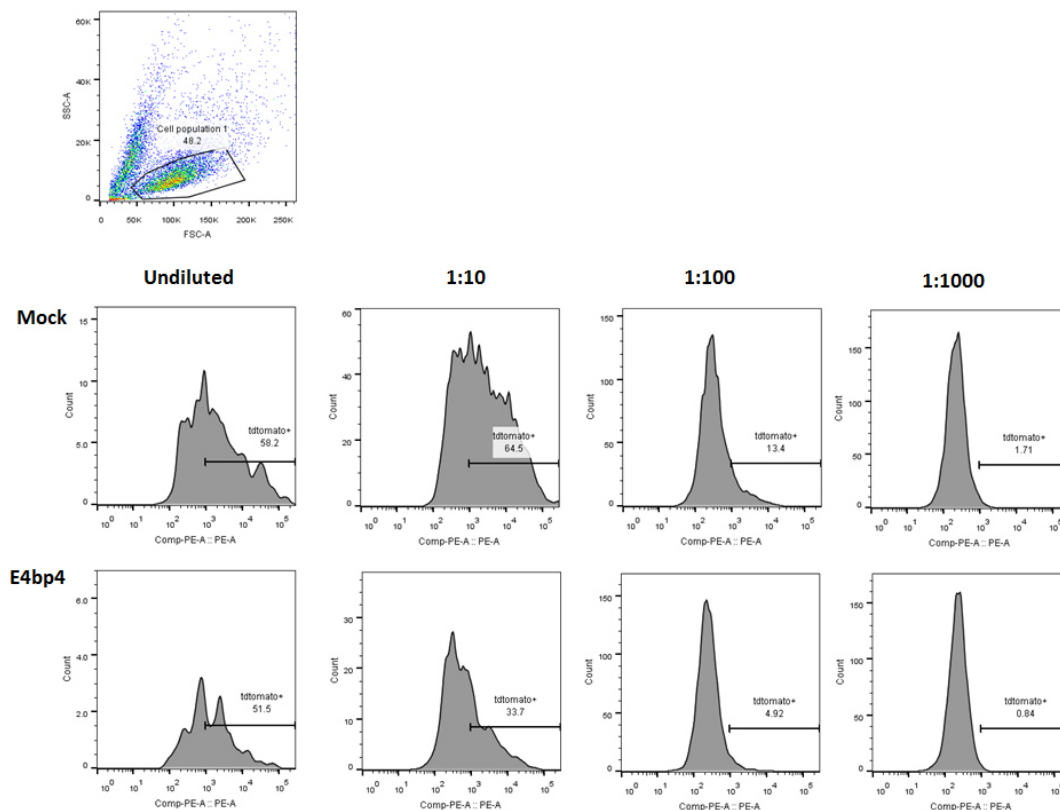
E4bp4

5 $\mu$ l of viral supernatant .....34% of 5x10<sup>4</sup> HEK293T cells

1000 $\mu$ l of viral supernatant .....34% of 1x10<sup>7</sup> HEK293T cells

= 3.4x10<sup>6</sup> infectious units/ml

Virus titer of E4bp4 lentivirus was almost tenfold lower than mock lentiviral prep.



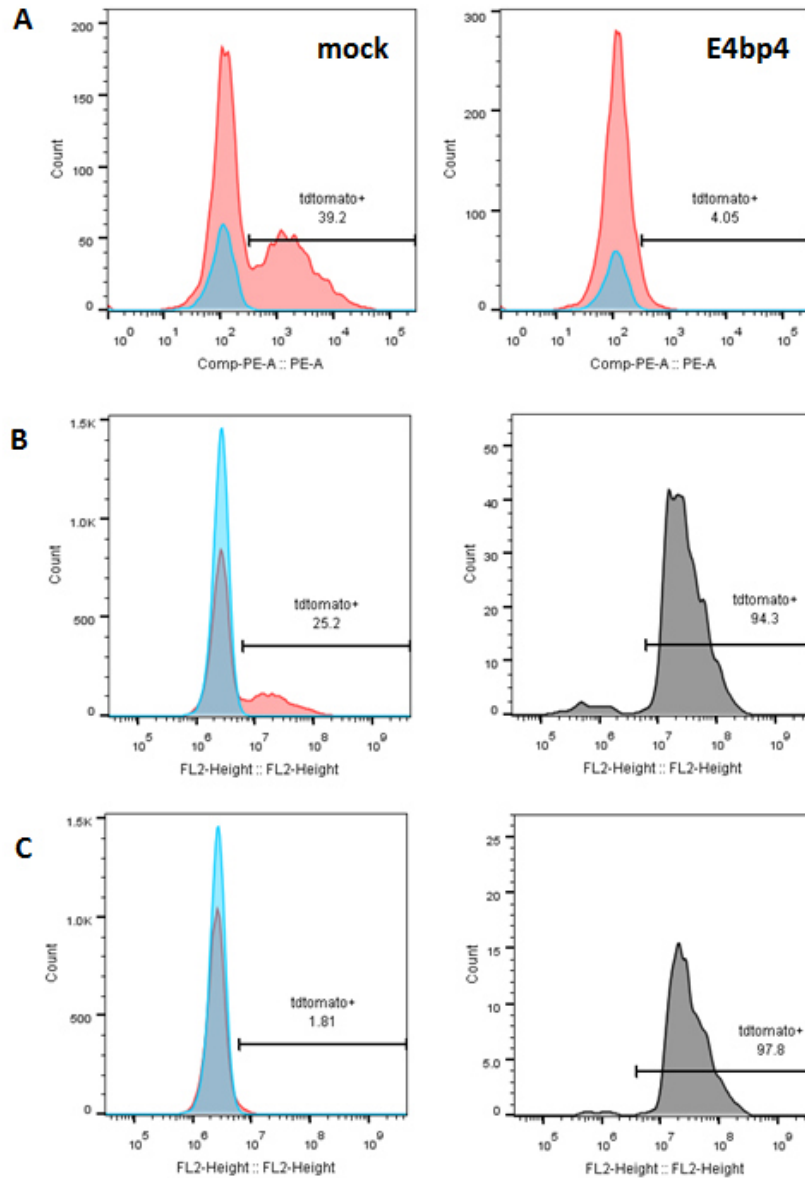
**Figure 5.4 Virus titration by HEK 293T transduction efficiency.** Tdtomato expression in cells transduced with varying concentrations of virus was determined by flow cytometry.



### 5.2.2 UCB-derived CD34<sup>+</sup> cells can be efficiently transduced

In the initial experiment we aimed to transduce UCB-derived CD34<sup>+</sup> cells to assess transduction efficiencies and if the expression of the construct can be maintained over the period of NK cell differentiation in GBGM medium.

UCB-derived CD34<sup>+</sup> cells were transduced with mock (pcSGW-IRES-tdtomato) and E4bp4 (pcSGW-hE4bp4-IRES-tdtomato) lentivirus, respectively, with MOI 5. Transduced and untransduced cells were cultured following the GBGM NK cell differentiation. On day 6 post-transduction, tdtomato expression was determined by flow cytometry. Transduction with mock lentiviral particles resulted in good efficiency of 39% of cells expressing tdtomato, compared to low transduction efficiency with 4% tdtomato-expressing cells after E4bp4 transduction (Figure 5.5 A). Due to very low transduction efficiency with the E4bp4 lentivirus, we decided to sort for tdtomato<sup>+</sup> cells by FACS on day 13. Mock transduced cells expressed 25% tdtomato on day 13 pre-sorting which could be enriched to a cell population expressing 94% (Figure 5.5 B). The E4bp4 transduced cell population expressed tdtomato only in 1.8% of cells which could be enriched to 98% post-sorting (Figure 5.5 C). However, due to the very low expression rate, the sorted cell population only consisted of 2000 cells which made further cell culture challenging.



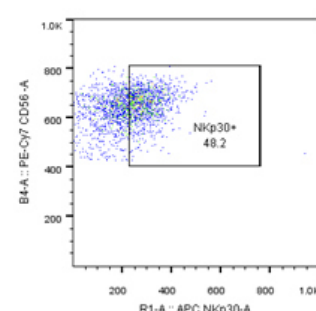
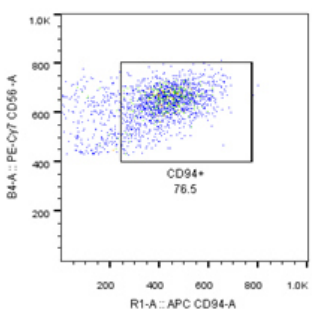
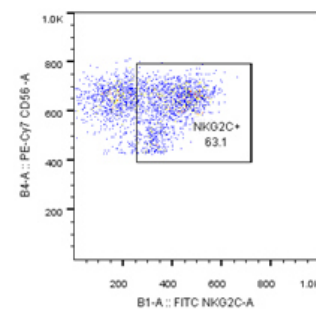
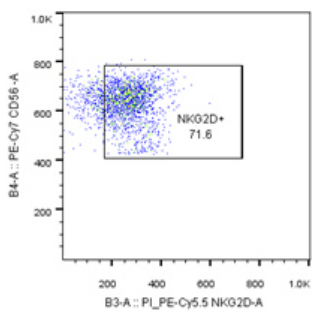
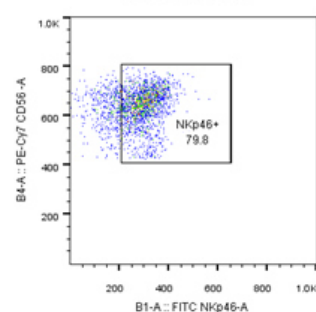
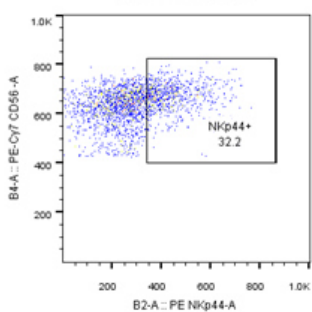
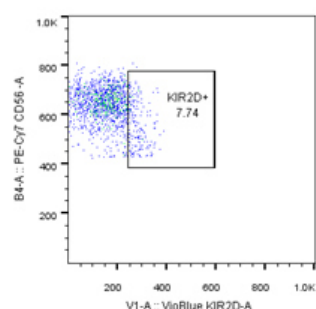
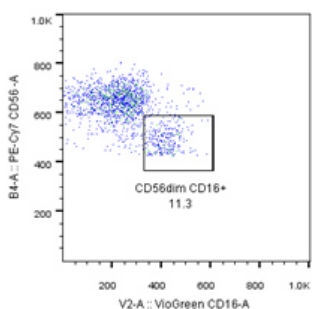
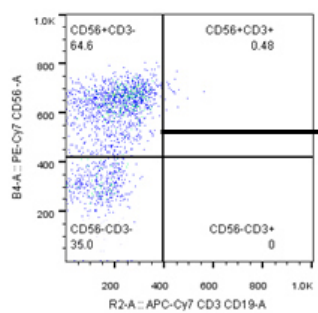
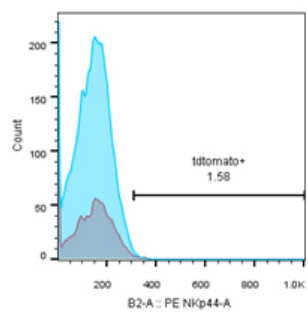
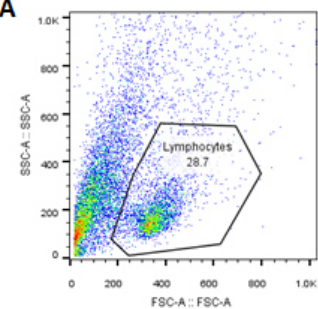
**Figure 5.5 Transduction efficiency of UCB-derived CD34<sup>+</sup> cells.** Tdtomato expression was determined by flow cytometry on day 6 post transduction with (A) mock vector (left) and E4bp4 vector (right). (B) Tdtomato expression on mock transduced cells on day 13 pre-sorting (left) and post-sorting (right) and (C) Tdtomato expression on E4bp4 transduced cells on day 13 pre-sorting (left) and post-sorting (right).

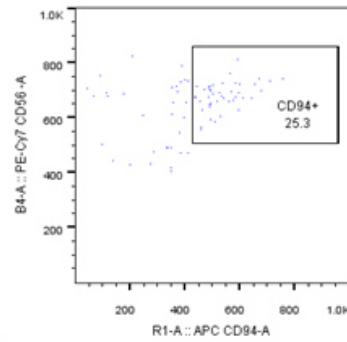
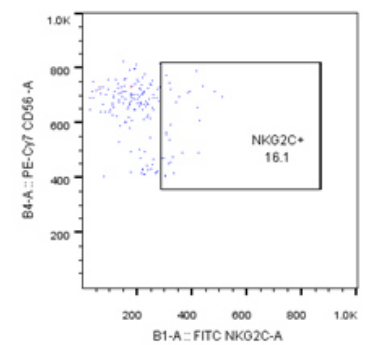
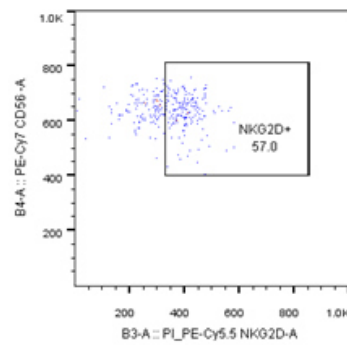
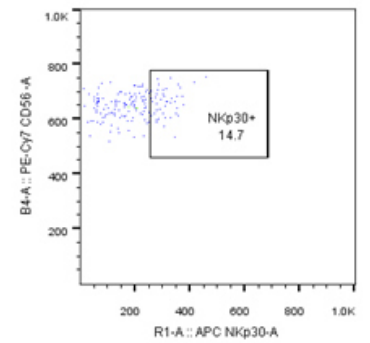
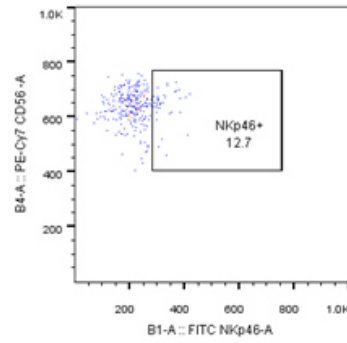
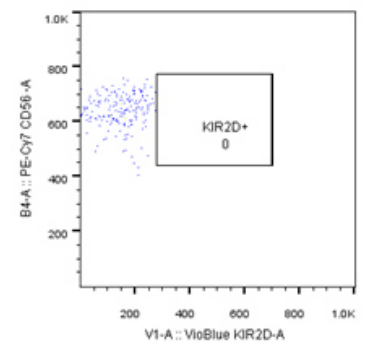
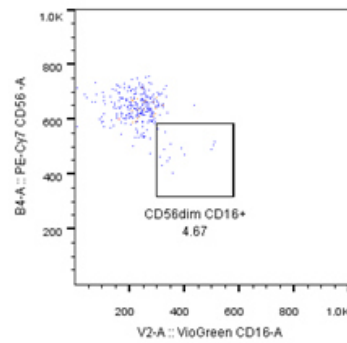
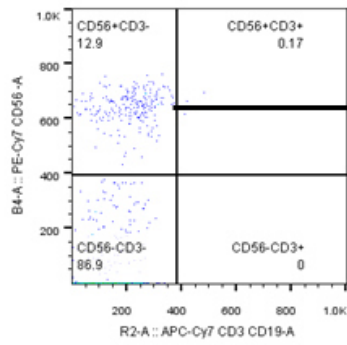
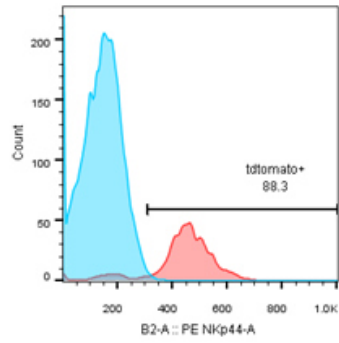
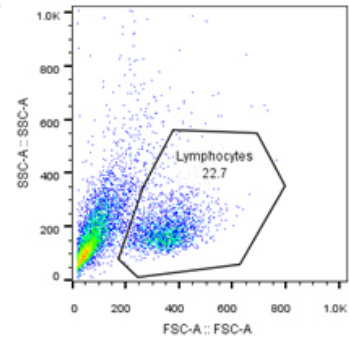
### **5.2.3 Lentiviral transduced UCB-derived NK cells keep tdtomato expression upregulated during NK cell differentiation and surface marker expression differs compared to untransduced UCB-derived NK cells**

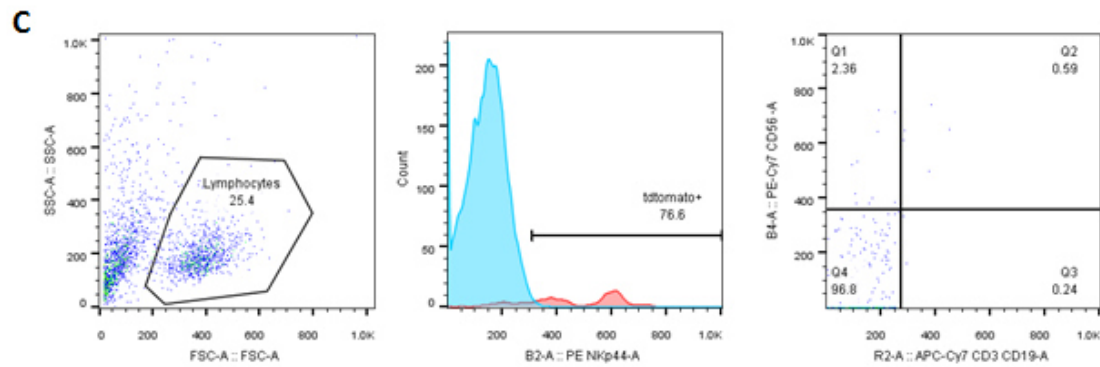
To investigate if mock and E4bp4 transduced UCB-derived NK cells express NK cell specific surface makers to similar levels compared to untransduced UCB-derived NK cells, we analysed GBGM-derived cells in parallel by multi-colour flow cytometry on day 27. Cells were gated on the CD56<sup>+</sup>CD3<sup>-</sup> NK cell population (64%) and transduction efficiency is shown in the histogram (Figure 5.6). Untransduced UCB-derived NK cells on day 27 display CD56<sup>bright</sup> and CD56<sup>dim</sup> sub-populations with 11% CD16 expressing cells (Figure 5.6 A). Compared to untransduced cells, CD56<sup>+</sup>CD3<sup>-</sup> NK cell population in mock transduced cells comprised of only 13% (Figure 5.6 B) and surprisingly of only 2% in E4bp4 transduced cells (Figure 5.6 C). The low NK cell generation could be due to very low cell numbers sorted which could make the NK cell differentiation less efficient. On the other side, E4bp4 could exhibit the opposite effect as proposed and inhibit NK cell differentiation.

The analysis of NK cell specific surface markers revealed a general lower expression profile in mock transduced cells. 7% of untransduced UCB-derived NK cells express KIR2D, compared to no expression in mock transduced cells (Figure 5.6 A and B). CD94 is expressed in 76% of untransduced and only in 25% of mock transduced cells. Lower expression could also be observed in NKp46, NKp30, NKG2D and NKG2C in mock transduced cells (Figure 5.6 A and B). NK cell marker expression could not be analysed in E4bp4 transduced cells because of low CD56<sup>+</sup>CD3<sup>-</sup> cell numbers.

**A**



**B**



**Figure 5.6 *In vitro* generated NK cells from UCB-derived CD34<sup>+</sup> cells on day 27 of feeder- and serum free GBGM differentiation method.** (A) untransduced and (B) mock transduced cells were analysed by multi-colour flow cytometry using a panel of antibodies for NK cell specific surface markers. Cells for surface marker analysis were gated on the CD56<sup>+</sup>CD3<sup>-</sup> cell population. The percentage of GFP expressing cells is represented in histograms (C) E4bp4 transduced cells could not be analysed for NK cell specific markers due to low CD56<sup>+</sup>CD3<sup>-</sup> cell population. All gates are set based on corresponding isotype controls.

### 5.2.4 Cloning of new lentiviral vector into pCCL.EF1 $\alpha$ .eGFP backbone

Because of the low lentiviral titer achieved with pSGW constructs, we decided to clone E4bp4 into a 3<sup>rd</sup> generation pCCL lentiviral backbone. This construct expresses GFP instead of tdtomato under the EF1 $\alpha$  promotor (Figure 5.7). MP12840.pCCL.EF1 $\alpha$ .eGFP was used as mock control. The correct size of the cloned and amplified E4bp4 overexpression plasmid was confirmed by restriction digest with SmaI and BstBI and expected DNA fragments of 8800bp and 1700bp could be observed in DNA from mini preps b-e (Figure 5.8). Lentivirus was produced with both the mock control and E4bp4 overexpression plasmid DNA from mini prep d.

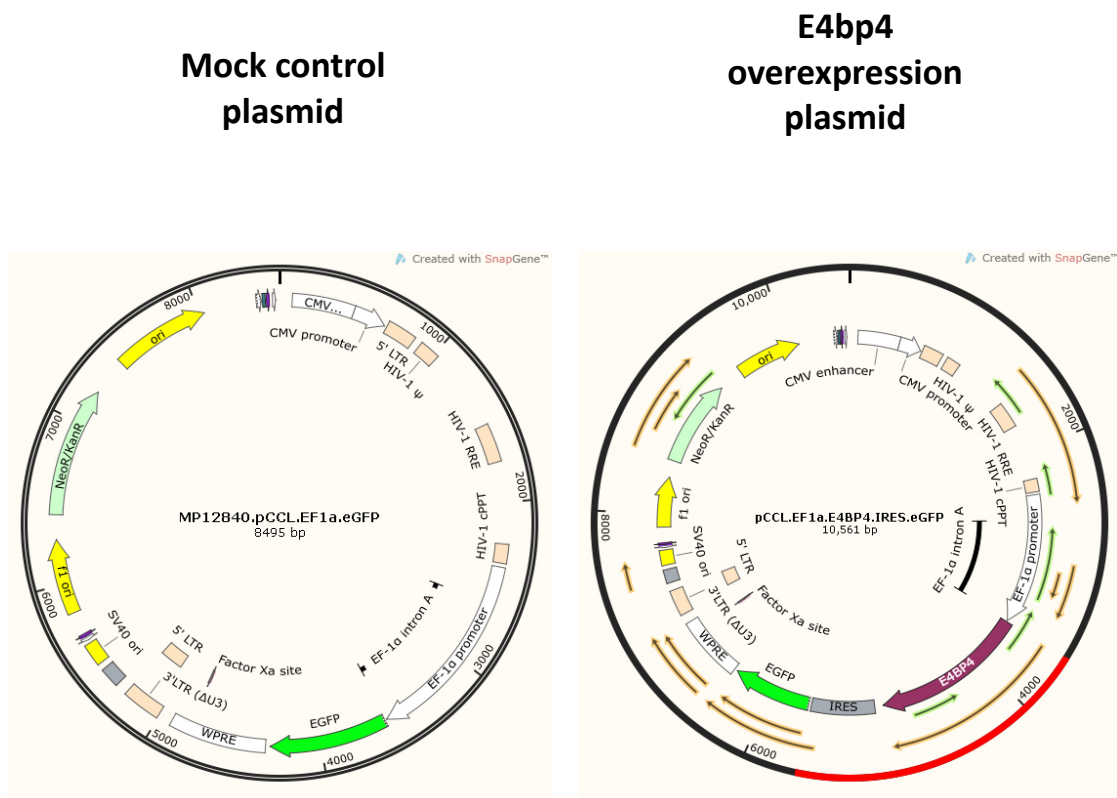
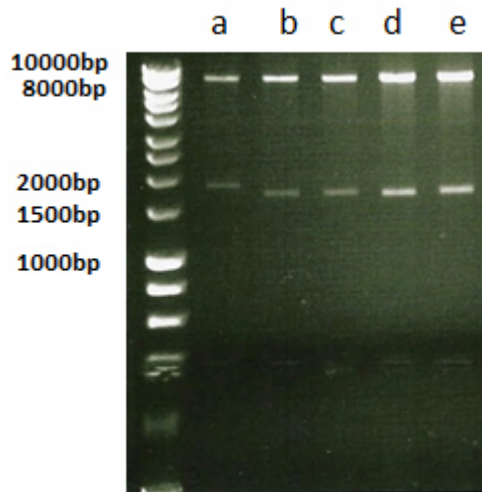


Figure 5.7 Plasmid maps of newly cloned lentivirus over-expression vectors.



**Figure 5.8 Restriction digest of E4bp4 overexpression plasmid DNA with SmaI and BstBI.** Correct sizes of DNA fragments could be observed in DNA from mini-preps b-e with 8800bp and 1700bp. Mini-prep d was chosen for further lentivirus production. A 1kb DNA ladder was used in gel electrophoresis.

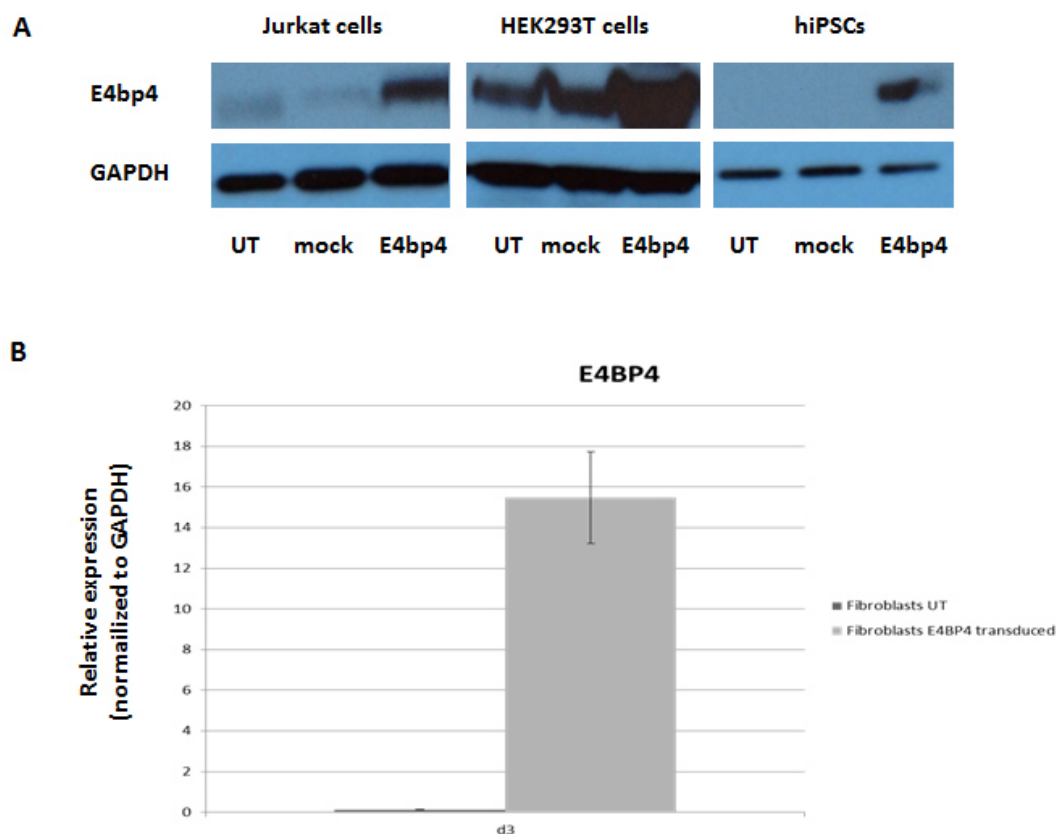
The lentiviral titer was determined by transduction of HEK293T cells with various concentrations of virus. A higher titer could be achieved with the pCCL lentiviral constructs: mock  $4.9 \times 10^9$  IU/ml and E4bp4  $2 \times 10^7$  IU/ml (data not shown).

#### **5.2.5 E4bp4 over-expression with pCCL.EF1 $\alpha$ .E4bp4.IRES.eGFP can be detected on RNA and protein level**

To test the upregulation of E4bp4 on RNA and protein level, we transduced Jurkats, HEK293T cells and hiPSCs with either mock or E4bp4 over-expression lentivirus. Western blot analysis showed the detection of E4bp4 in all three cell lines transduced with E4bp4 lentivirus (Figure 5.9 A). E4bp4 is endogenously expressed



in Jurkats and HEK293T cells, which was detected in untransduced and mock transduced cells. Overexpression could be observed in E4bp4 transduced cells. Upregulation could also be detected on RNA level with E4bp4 transduced fibroblasts (Figure 5.9 B).



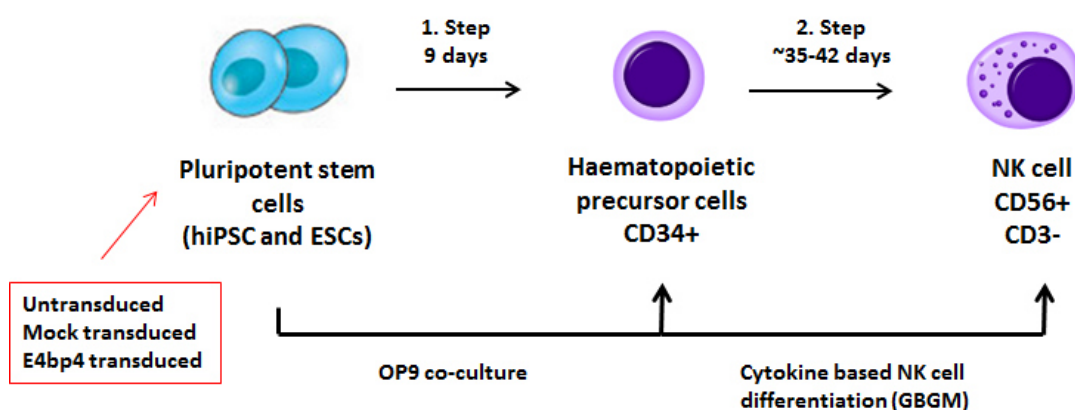
**Figure 5.9 Lentivirus construct successfully upregulates E4bp4 in Jurkat cells, HEK293T and hiPSCs.** (A) E4bp4 (60 kDa) over expression in Western Blot with GAPDH loading control and (B) relative expression (normalized to GAPDH) determined by RT-qPCR in fibroblasts. Results are presented as Mean±SD, n=3 technical replicates.

### **5.2.6 Direct differentiation of hiPSCs to NK cells by over-expression of E4bp4**

Our initial experiment aimed to over-express E4bp4 in hiPSCs to attempt direct differentiation into the NK cell lineage. We transduced MMC hiPSCs with the mock (pCCL.EF1 $\alpha$ .eGFP) and E4bp4 (pCCL.EF1 $\alpha$ .E4bp4.IRES.eGFP) lentivirus, respectively. Directly after transduction, cells were transferred into NK cell differentiation conditions with GBGM medium and corresponding cytokines. Cell morphology and cell numbers were monitored. Cell viability decreased radically after a few days in culture and no cell characterization could be performed. Haematopoietic, and further NK cell development, is a long process requiring sequential acquisition of receptors, important for recognition of maturation signals of the environment. We concluded that the expression of a single NK cell-specific TF in combination with NK cell differentiation specific culture conditions is not sufficient to induce a transcriptional network that facilitates the maturation of NK cells. Therefore, we decided to over-express E4bp4 in the established 2-step differentiation method to investigate if a combination of cytokine- and TF-driven differentiation can improve NK cell differentiation and/or phenotype.

### **5.2.7 Can the over-expression of E4bp4 in hiPSCs improve the efficiency of NK cell differentiation?**

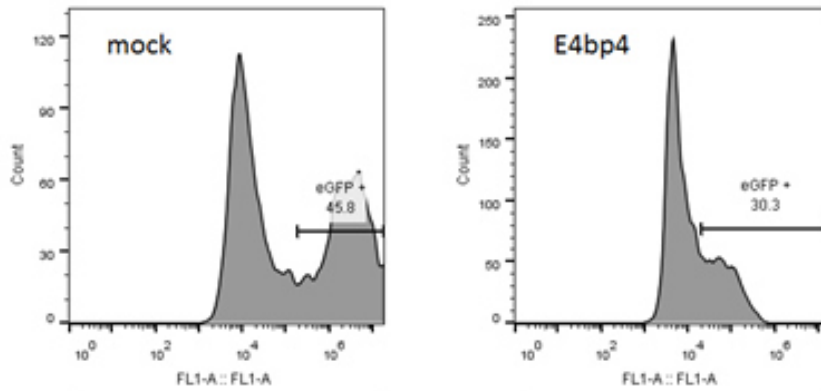
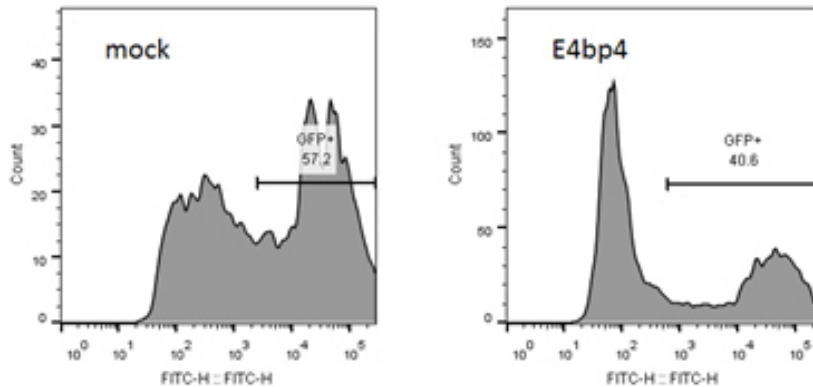
MMC hiPSCs were transduced with mock (pCCL.EF1 $\alpha$ .eGFP) and E4bp4 (pCCL.EF1 $\alpha$ .E4bp4.IRES.eGFP), respectively, with MOI 20. Transduced cells were expanded in pluripotent stem cell conditions using mTESR1, differentiated towards CD34<sup>+</sup> haematopoietic precursors with OP9 co-culture, MACS enriched and differentiated to NK cells with GBGM culture conditions (Figure 5.10).



**Figure 5.10 Schematic overview of experimental layout – E4bp4 overexpression in 2-step NK cell differentiation from hiPSCs.**

### **5.2.8 Good transduction efficiency of hiPSCs and GFP upregulation is maintained during cell expansion in pluripotent culture conditions**

Transduction efficiency was determined by GFP expression with flow cytometry on day 3 post-transduction. 46% of mock and 30% of E4bp4 transduced cells expressed GFP (Figure 5.11 A). Transduction efficiency with pCCL lentivirus was more efficient compared to transductions with the original pcSGW lentivirus. After 3 passages in pluripotent stem cell conditions with mTESR1, the transduction efficiency increased to 57% of GFP expressing cells with mock and 40% GFP expressing cells with E4bp4 transductions (Figure 5.11 B). These cells represent day 0 of the differentiation experiment.

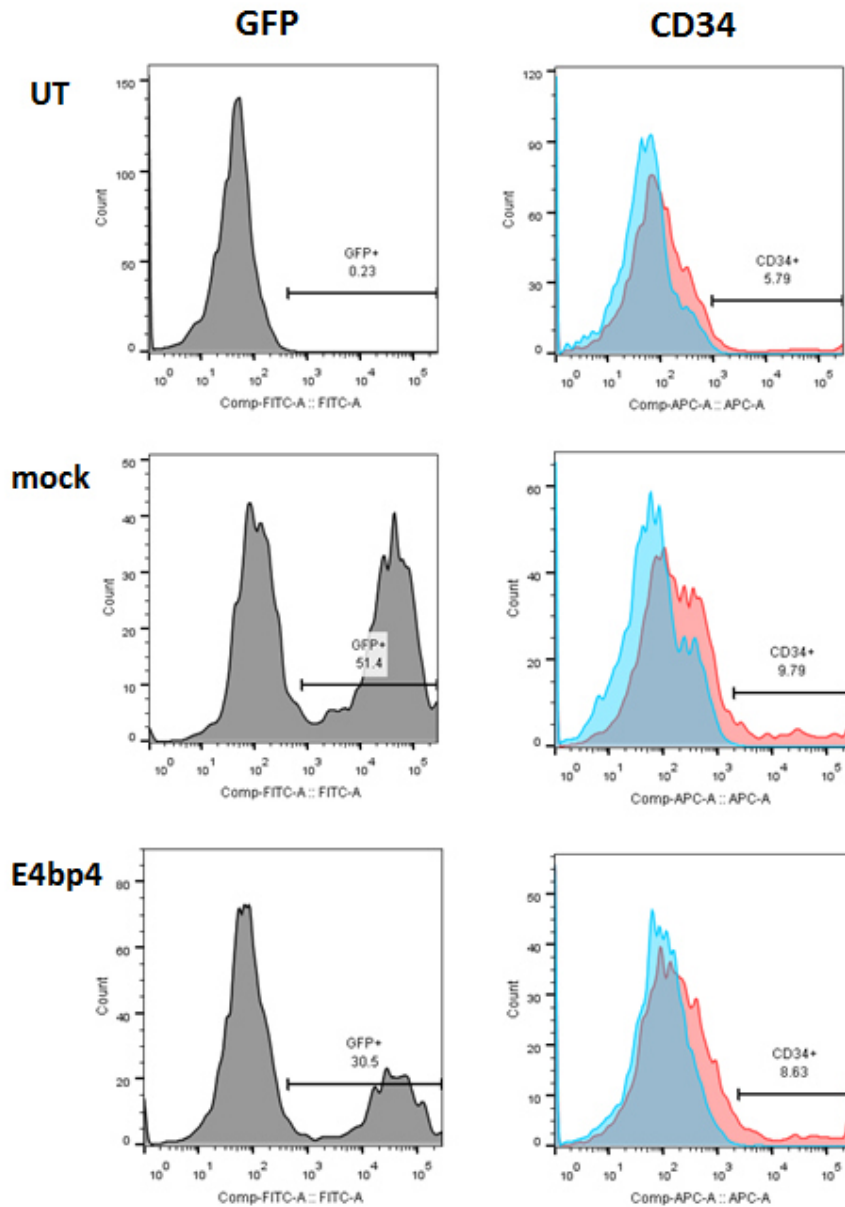
**A****Day 3****B****After 3 passages = d0**

**Figure 5.11 MMC hiPSC transduction efficiency.** GFP expression was determined by flow cytometry on (A) day 3 post-transduction and (B) after 3 passages, representing day 0 of the differentiation experiment. Cells were kept in pluripotent culture conditions with mTESR1.

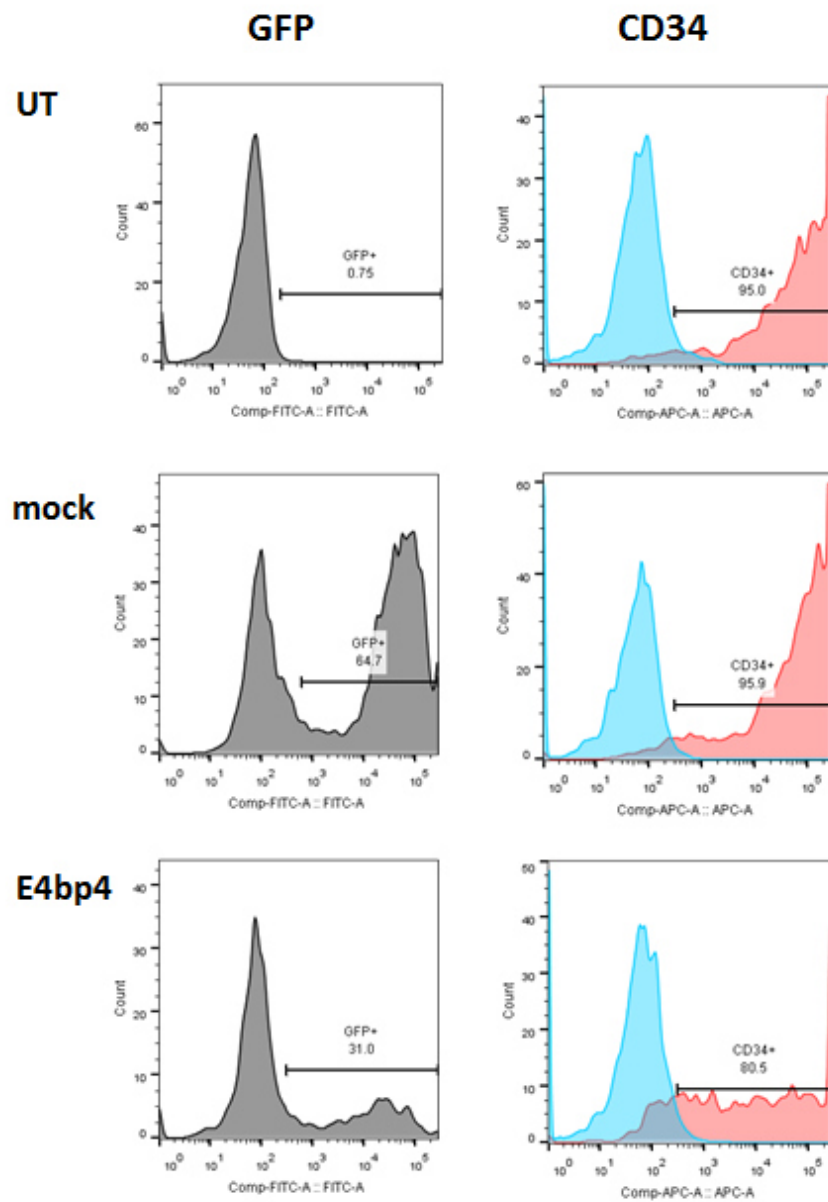
### 5.2.9 CD34<sup>+</sup> cell derivation from transduced hiPSCs with OP9 co-culture

HPSC-derived CD34<sup>+</sup> cells were generated with OP9 co-culture with the following differentiation efficiencies: untransduced 5.8%, mock transduced 9.8% and E4bp4 transduced 8.6% CD34<sup>+</sup> expressing cells (Figure 5.12). GFP expression slightly decreased to 51% in mock and 30.5% in E4bp4 transduced cells. CD34<sup>+</sup> cells were enriched by CD34 MACS technology. The following purities of CD34<sup>+</sup> expressing

cells could be achieved: untransduced 95%, mock transduced 96% and E4bp4 transduced 80% (Figure 5.13).



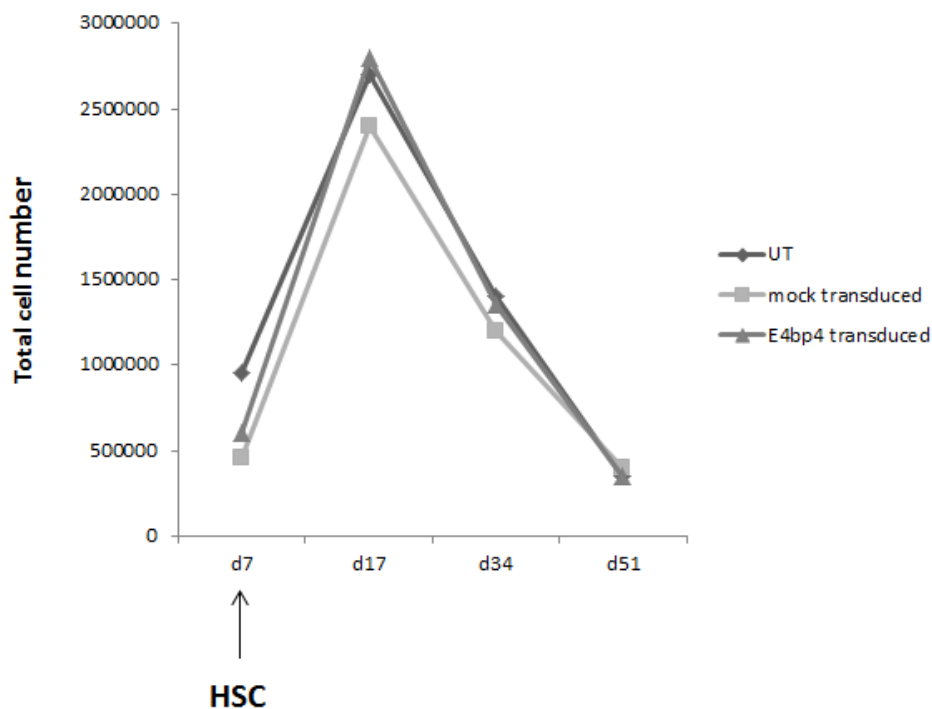
**Figure 5.12 CD34 expression efficiency on day 9 of OP9 co-culture differentiation.** GFP and CD34 expression was determined by flow cytometry in hiPSCs untransduced, mock and E4bp4 transduced cells. Blue histograms represent the isotype control staining and red histograms the specific staining.



**Figure 5.13 CD34 MACS enrichment efficiency of OP9 co-culture derived CD34<sup>+</sup> cells.** GFP and CD34 expression was determined by flow cytometry in hiPSCs untransduced, mock and E4bp4 transduced cells. Blue histograms represent the isotype control staining and red histograms the specific staining.

### 5.2.10 E4bp4 overexpression does not improve cell expansion in NK cell differentiation

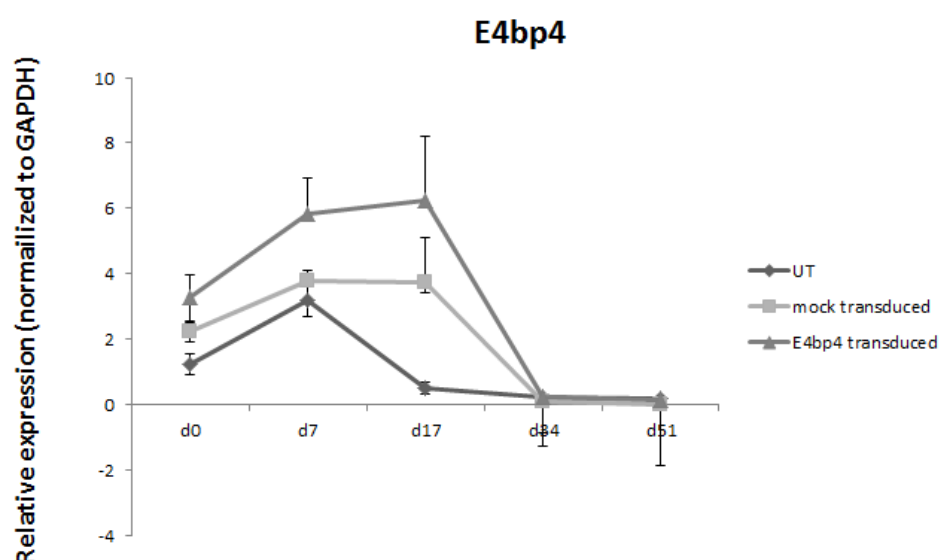
To determine any improvements in the cell expansion potential of E4bp4 transduced hiPSCs in GBGM differentiation, we documented total cell counts during *in vitro* NK cell generation (Figure 5.14). Our data suggest a slightly higher expansion of E4bp4 transduced cells within the first 10 days of culture when compared to untransduced and mock transduced cells (Figure 5.14). However, the maximum expansion did not exceed 5 fold with cell numbers decreasing significantly within the last 3 weeks of culture.



**Figure 5.14 Total cell numbers during NK cell differentiation.** Total cell numbers of untransduced, mock and E4bp4 transduced cells were determined on day 7, 17, 34 and 51 during differentiation. Day 7 represents the CD34<sup>+</sup> HSC stage.

### 5.2.11 E4bp4 is consistently upregulated during NK cell differentiation

To investigate if E4bp4 is overexpressed in E4bp4 transduced cells during differentiation, we collected RNA samples on days 0, 7, 17, 34 and 51 of *in vitro* NK cell generation in GBGM culture conditions. Relative expression of E4bp4 was determined by qRT PCR analysis, normalized to GAPDH. E4bp4 was overexpressed in E4bp4 transduced cells until day 34 in culture (Figure 5.15).



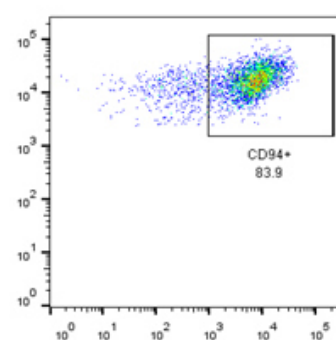
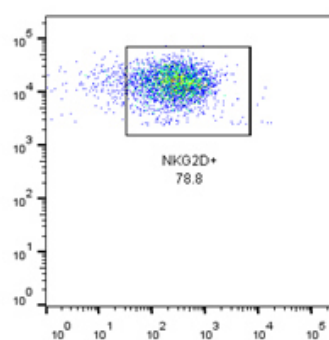
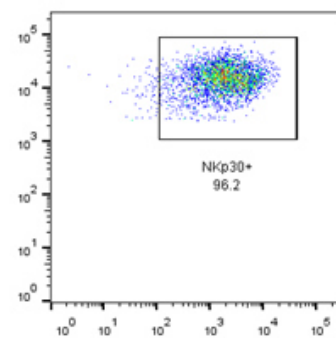
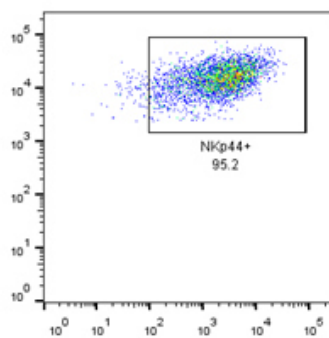
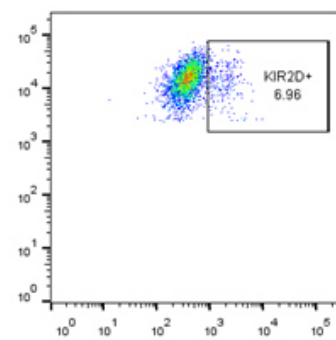
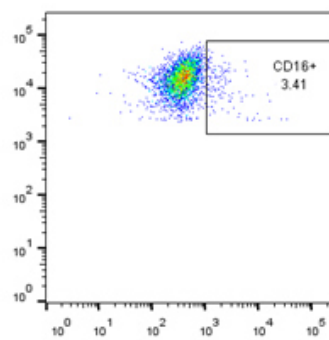
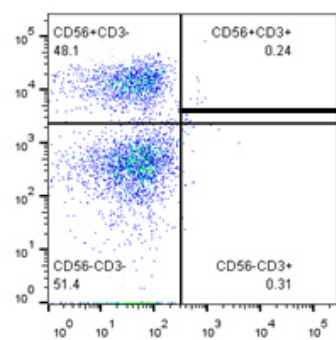
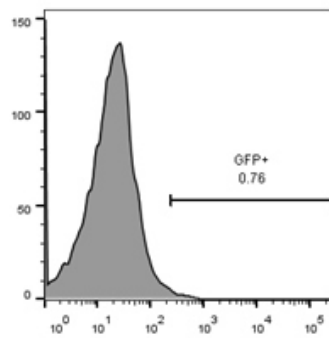
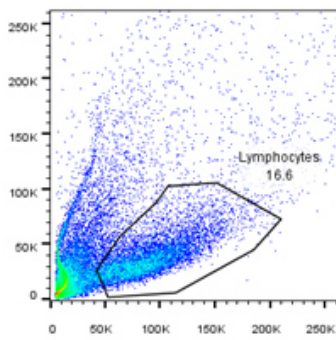
**Figure 5.15 Relative expression of E4bp4 during *in vitro* NK cell differentiation.** The relative expression of E4bp4 and Id2 was determined by RT-qPCR on day 0, 7, 17, 34 and 51 in cells of a 2-stage NK cell differentiation *in vitro*. Results are presented as Mean $\pm$ SD, n=3 technical replicates.

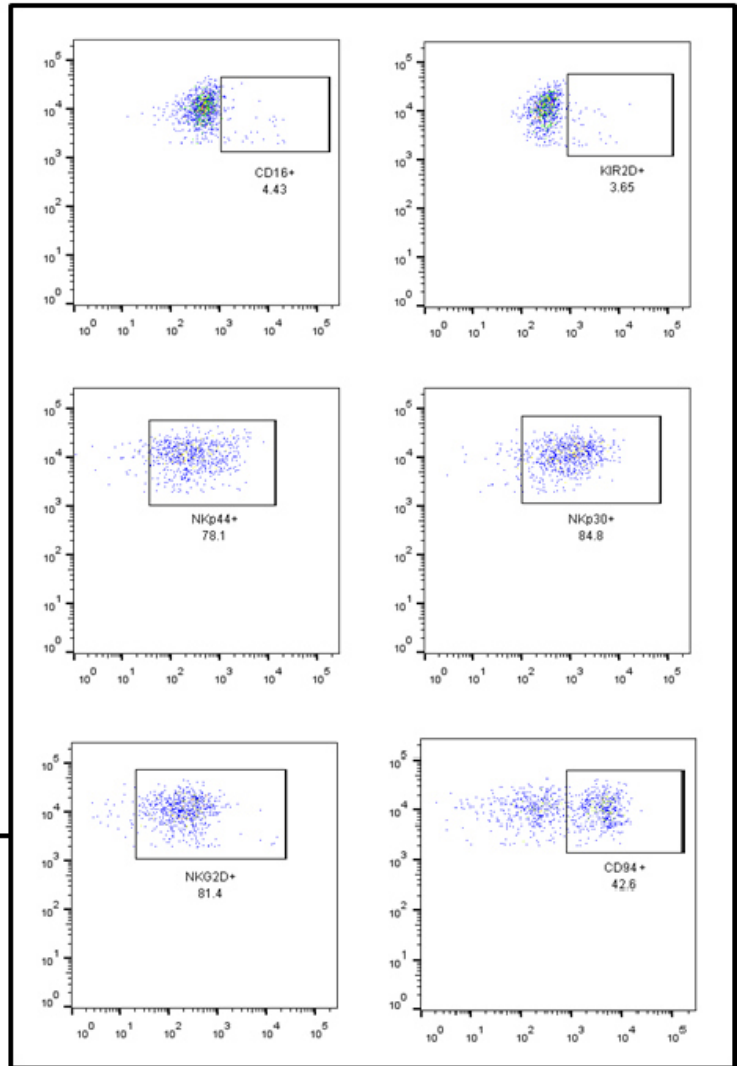
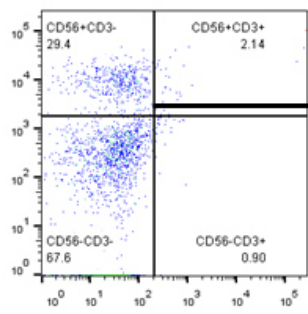
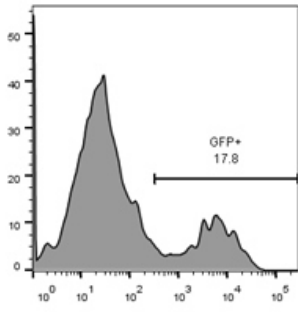
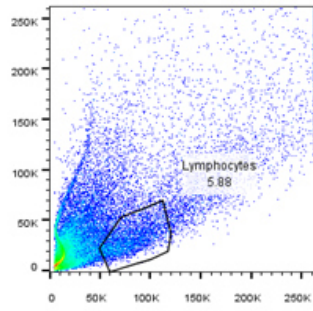


### **5.2.12 E4bp4 transduced hiPSC-derived NK like cells differ in surface marker expression compared to untransduced and mock transduced cells**

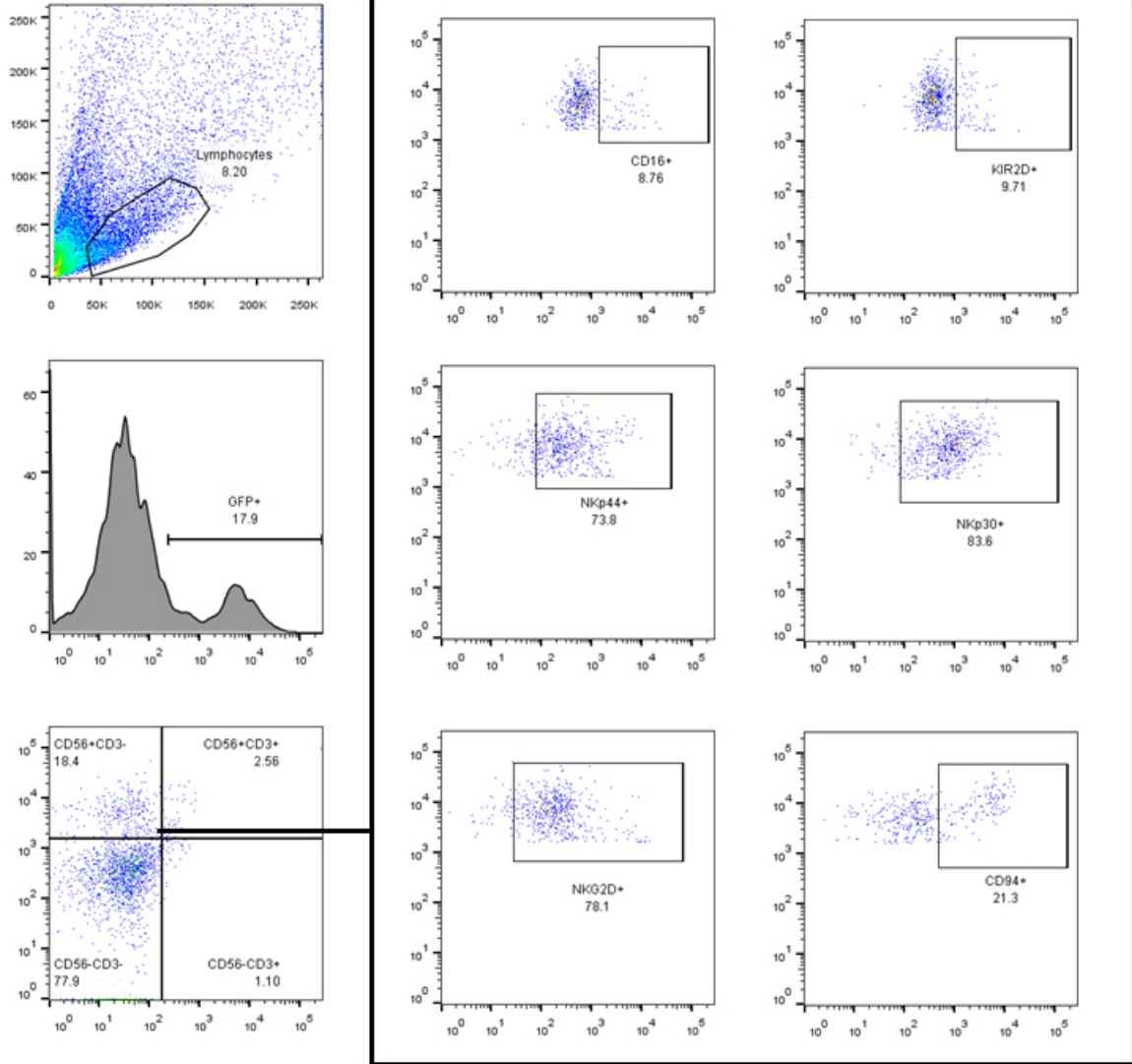
To investigate if E4bp4 transduced hiPSC-derived NK like cells express NK cell specific surface makers to similar levels compared to untransduced and mock transduced cells, we analysed GBGM-derived cells in parallel by multi-colour flow cytometry on day 51. Cells were gated on the CD56<sup>+</sup>CD3<sup>-</sup> NK cell population and transduction efficiency is shown in histograms (Figure 5.16). Untransduced cells comprised of 48% CD56<sup>+</sup>CD3<sup>-</sup>, compared to 29% in mock transduced and 18% in E4bp4 transduced cells which was generally lower to what was observed in chapter 4. Distinct CD56<sup>bright</sup> and CD56<sup>dim</sup> sub-populations could not be observed in hiPSC-derived NK like cells. Interestingly, CD16 expression was highest in E4bp4 transduced cells with 9% compared to 4% and 3% in mock transduced and untransduced cells, respectively. Furthermore, higher panKIR2D expression of 10% could be observed in E4bp4 transduced NK like cells, compared to 4% and 7% in mock transduced and untransduced cells, respectively. In contrast, CD94 expression was highest in untransduced cells with 84%, 43% of mock transduced cells expressed CD94 and only 21% of E4bp4 transduced NK like cells.

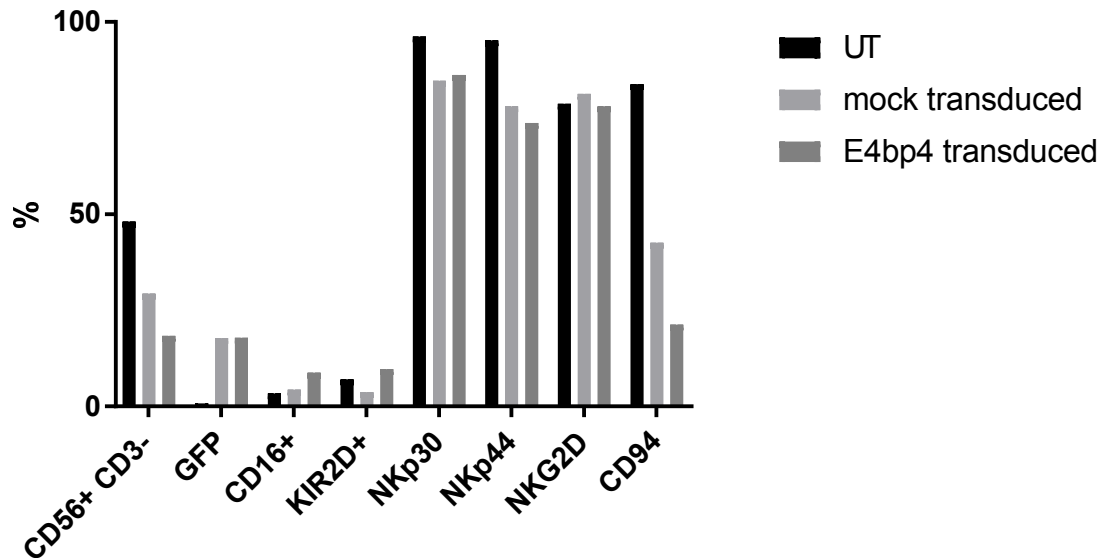
The NCR marker NKp44 and NKp30 expression was slightly lower in both mock and E4bp4 transduced cells compared to the untransduced control. NKG2D expression could be observed to a similar level in all three cell lines (Figure 5.16).

**A**

**B**

C



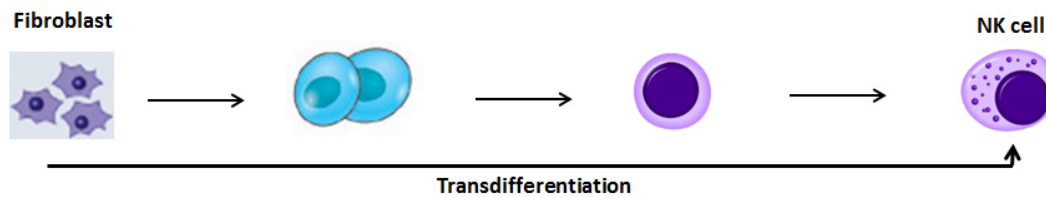


**Figure 5.16** *In vitro* generated NK cells from MMC hiPSC-derived CD34<sup>+</sup> cells on day 51 of feeder- and serum free GBGM differentiation method. (A) untransduced, (B) mock transduced, and (C) E4bp4 cells were analysed by multi-colour flow cytometry using a panel of antibodies for NK cell specific surface markers. Cells for surface marker analysis were gated on the CD56<sup>+</sup>CD3<sup>-</sup> cell population. The percentage of GFP expressing cells is represented in the histogram. (C) Expression data in % are summarised in a bar chart, n=1.

### 5.2.13 Can fibroblasts be driven towards the NK cell lineage by over-expression of E4bp4 and culture in GBGM NK cell differentiation conditions?

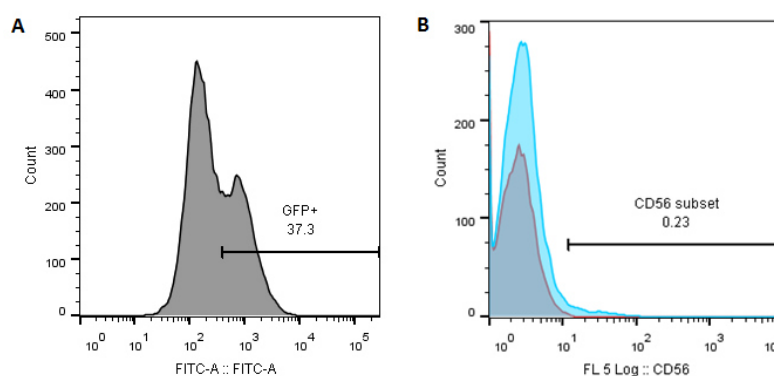
We aimed to investigate if the over-expression of E4bp4 - as master-regulator of the transcriptional NK cell program - can induce cells to acquire an NK cell like phenotype. To test this hypothesis, we transduced human fibroblasts with

pCCL.EF1 $\alpha$ .E4bp4.IRES.eGFP lentivirus with MOI 20 and cultured cells in GBGM medium containing cytokines supporting NK cell development. Transdifferentiation of somatic cells would allow to by-pass the reprogramming to hiPSCs and the limiting differentiation step to HSCs (Figure 5.17).



**Figure 5.17 Schematic overview of transdifferentiation.**

Transduction efficiency of fibroblasts was assessed by the expression of GFP with flow cytometry 3 days post-transduction, cultured in fibroblast medium. A good proportion of cells had been transduced with 37% of cells expressing GFP. CD56 was not expressed in fibroblasts (Figure 5.18).

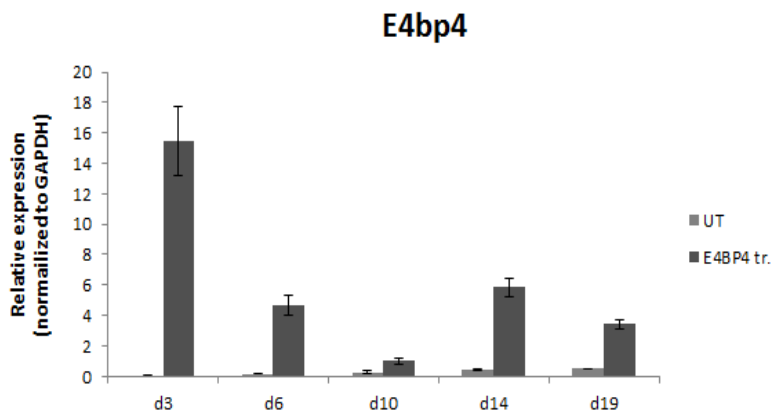


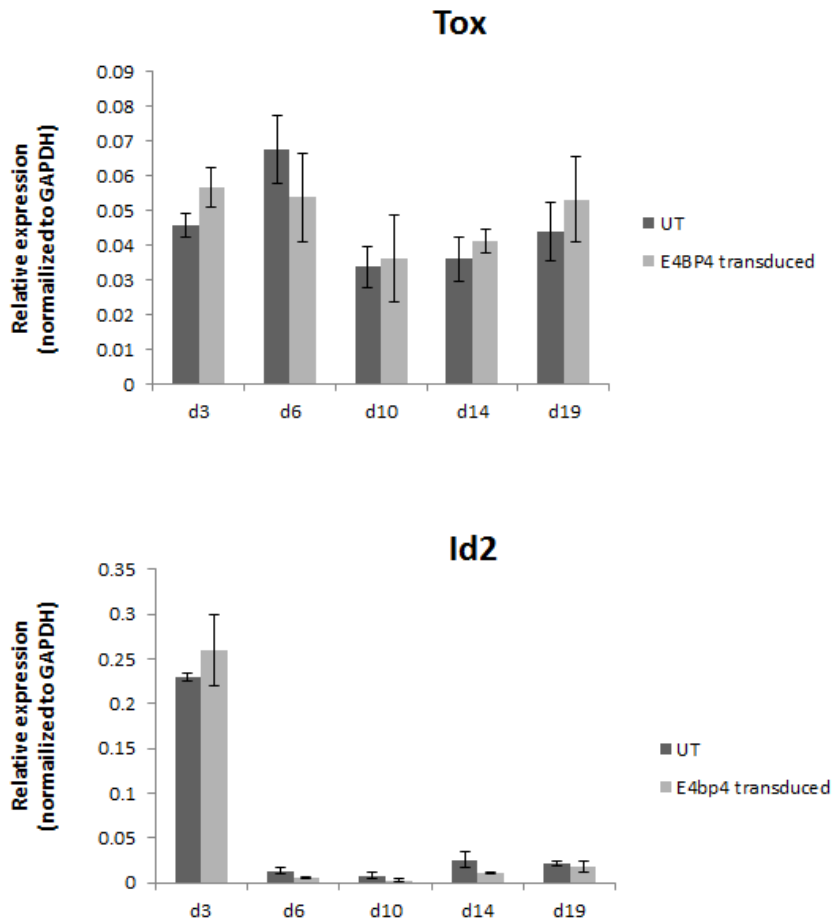
**Figure 5.18 Transduction efficiency day 3 post-transduction of fibroblasts. (A) GFP expression (B) CD56 expression.**

#### 5.2.14 E4bp4 stays upregulated during cell culture

On day 3 post-transduction, fibroblasts were cultured in conditioned medium containing half fibroblast medium and half GBGM Dif1 medium containing SCF, IL-7, Flt3L, IL-15, G-CSF, GM-CSF and IL-6. On day 6 the medium ratio was changed to 25% fibroblast and 75% GBGM Dif1 medium and on day 12 to 100% GBGM medium containing SCF, IL-7, IL-15, IL-2, G-CSF, GM-CSF and IL-6. RNA samples were collected on days 3, 6, 10, 14 and 19 of culture. E4bp4 over-expression in pCCL.EF1 $\alpha$ .E4bp4.IRES.eGFP-transduced cells could be observed throughout culture when compared to untransduced cells with highest expression on day 3. (Figure 5.19).

To investigate if the transcriptional network is activated by the over-expression of E4bp4, we followed the expression levels of Tox and Id2 which have been shown to be regulated by E4bp4 (Male, Nisoli et al. 2014). No significant up-regulation of Tox or Id2 could be observed when compared to untransduced cells (Figure 5.19). These data suggest that the over-expression of E4bp4 is not sufficient to induce the NK cell specific transcriptional network in fibroblasts.





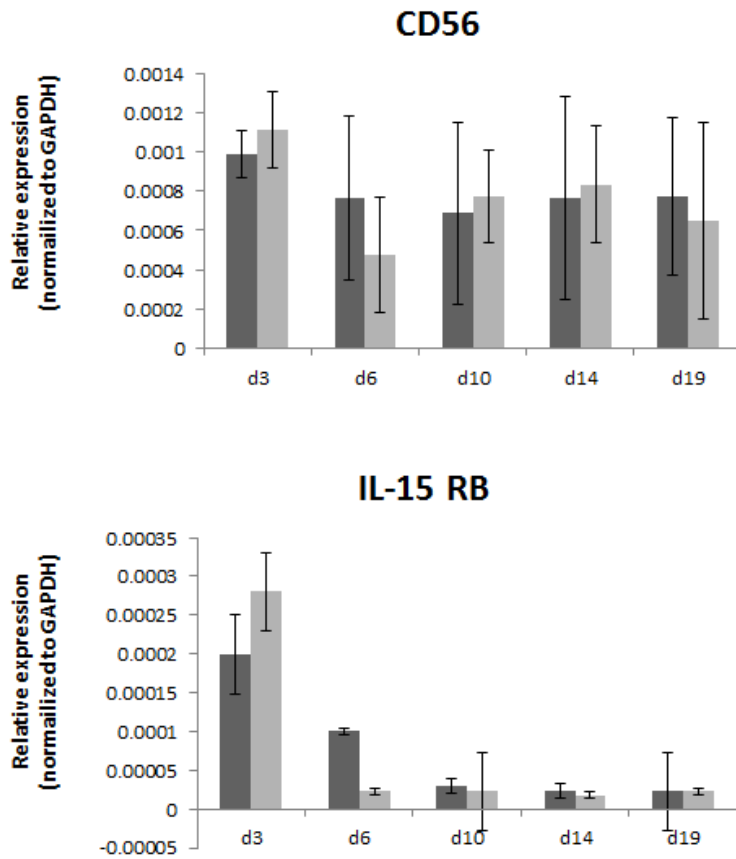
**Figure 5.19 Relative expression of E4bp4, Tox and Id2 in fibroblasts over-expressing E4bp4 and cultured in NK cell differentiation medium.** The relative expression of E4bp4, Tox and Id2 was determined by RT-qPCR on day 3, 6, 10, 14 and 19, normalized to GAPDH. Results are presented as Mean $\pm$ SD, n=3 technical replicates.

#### 5.2.15 CD56 and IL-15RB are not upregulated in E4bp4 transduced cells

We further investigated if CD56 and IL-15RB are upregulated in E4bp4 transduced cells. IL-15RB is a subunit of the IL-15 receptor and its upregulation is a crucial step in the development of early NK cell precursors and makes progenitors responsive to IL-15 stimulation. The study of Male et al. suggests, that E4bp4 regulates IL-15RB

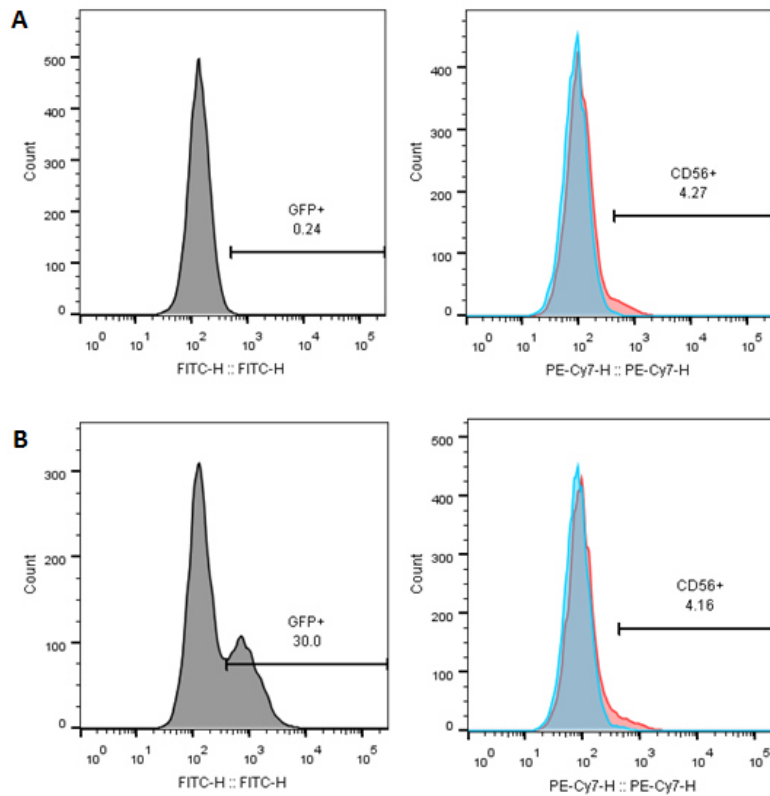


expression as one of the first steps to induced NK cell progenitors (Male, Nisoli et al. 2014). We could not observe either CD56 or IL-15RB upregulation in E4bp4 transduced (Figure 5.20).



**Figure 5.20 Relative expression of CD56 and IL-15 RB in fibroblasts over-expressing E4bp4 and cultured in NK cell differentiation medium.** The relative expression of CD56 and IL-15 RB was determined by RT-qPCR on day 3, 6, 10, 14 and 19, normalized to GAPDH. Results are presented as Mean $\pm$ SD, n=3 technical replicates.

To verify the CD56 expression on RNA level, we determined CD56 expression on the cell surface by flow cytometry on day 14 of culture. CD56 was expressed on 4% of both untransduced and E4bp4 transduced cells (Figure 5.21). These data suggest that no significant upregulation of CD56 was induced by E4bp4 overexpression.



**Figure 5.21 FACS analysis of GFP- and CD56-expressing cells on day 14 in adherent cells.** (A) Untransduced and (B) E4bp4 transduced cells. Blue histograms represent unstained control and red overlay the specific staining.

In summary our data suggest that the overexpression of E4bp4 does not upregulate the expression of Id2, TOX, CD56 and IL-15RB. We conclude that a single transcription factor might not be sufficient to induce NK cell lineage specific progenitors.

### 5.3 Discussion

In the first part of this chapter we investigated if the overexpression of the NK cell specific transcription factor E4bp4 can drive NK cell differentiation from hiPSCs more efficiently. We received the mock and E4bp4 overexpression lentiviral vector from a collaborator and produced lentivirus in HEK293T cells. Interestingly, we observed low virus titer with both constructs, although the titer with the E4bp4 vector was significantly lower than the titer with the mock vector. Despite the low virus titer, we could efficiently transduce UCB-derived CD34<sup>+</sup> cells with the mock lentivirus. Tdtomato reporter gene expression was still upregulated after 27 days of NK cell differentiation. However, transduction efficiency with the E4bp4 lentivirus was extremely low and the attempted sort did not yield enough transduced cells for further analysis. After preparing more virus preps with comparably low titer we needed to investigate why tdtomato in the E4bp4 lentivirus is less expressed. We believe that the low expression efficiency could be caused by the location of E4bp4 within the lentivirus plasmid. E4bp4 is located in front of an internal ribosome entry site (IRES) followed by tdtomato. IRES sites aim to improve the transcription of genes located further away from the promoter, but expression of tdtomato could still be hampered in this set up. Based on these observations, we decided to clone E4bp4 into the pCCL lentiviral vector backbone. Expression in this vector was driven by the EF1 $\alpha$  promoter and GFP was included as the reporter gene. Higher titers could be achieved with this construct for both, mock and E4bp4, although we still observed lower GFP expression with the E4bp4 overexpression virus. Produced virus could efficiently transduce Jurkat and HEK293T cells and overexpression of E4bp4 could be detected on both RNA and protein level. MMC hiPSCs could be efficiently transduced with both, mock and E4bp4 lentivirus, and cells could be expanded in their pluripotent state without losing expression from the lenti vector. This was an important observation because hiPSCs can be expanded well in the

pluripotent state which opens possibilities to bulk, freeze, sort transduced cells or even genetically modify further. Transduced MMC hiPSCs were differentiated with the established 2-step NK cell differentiation system, including OP9 co-culture, CD34 MACS enrichment and further differentiation with GBGM medium and cytokines. CD34<sup>+</sup> cells could be generated to comparable levels as observed in Chapter 3. We noticed lower purity of the CD34<sup>+</sup> enriched cell population transduced with E4bp4 compared to untransduced and mock transduced cells. This is possibly due to a variation in MACS enrichment as CD34 percentages before sorting were not significantly lower than observed in mock transduced cells. E4bp4 did not influence the cell expansion during NK cell differentiation and expansion in untransduced, mock transduced and E4bp4 transduced cells did not exceed 5 fold with cell numbers decreasing after 17 days of differentiation. E4bp4 upregulation could be detected throughout the differentiation on RNA level, although a drop of E4bp4 expression could be detected in all cell lines on day 34 and 51. We believe that this might be due to limited cell numbers collected for analysis. Despite the poor cell expansion we generated enough NK like cells which were analysed by a multi-colour flow cytometry panel after 51 days of differentiation. Consistent with our previous observations, NK like cells could not be classified as classical CD56<sup>dim</sup> and CD56<sup>bright</sup> sub-populations although interestingly, CD16 and panKIR2D expression and was highest in E4bp4 transduced cells. Surprisingly, CD94 expression was lower in E4bp4 transduced cells compared to mock transduced cells. I aimed to gate on the GFP<sup>+</sup> and GFP<sup>-</sup> populations to identify if transduced cells exhibit a different phenotype compared to mock transduced cells. This approach would have been extremely helpful to identify differences in marker expression, especially CD16 and KIR as these were expressed to higher levels in E4bp4 transduced cells. For this experiment a higher cell number would have been needed to be acquired as the low event count did not allow to perform an accurate analysis of GFP<sup>+</sup> or GFP<sup>-</sup> cells.

The percentage of transduced cells on day 51 of differentiation was 17%. Possibly, a phenotype is difficult to determine with less than a quarter of cells being transduced. For further experiments, cell sorting before differentiation would have been an option. In general, the interpretation of these results needs to be done with caution as repeated experiments would have been necessary. Unfortunately, due to time constraints this was not possible. Furthermore, we questioned if the established 2-step NK cell differentiation method was the best platform to study transcription factor driven differentiation, because of hurdles with cell numbers and expansion.

In the second part of this chapter we investigated if NK cells can be generated directly from fibroblasts by overexpression of E4bp4, without the need of reprogramming and subsequent forward differentiation. Transdifferentiation with a single transcription factor has been an ambitious aim. There are many prove-of-principle studies published on transdifferentiation into various cell types, reviewed in (Pfaff and Cantz 2013). However, in most cases, a combination of several TFs is necessary to activate the cell type specific transcriptional network. Since E4bp4 has been described as a master regulator acting very early in murine NK cell development (Male, Nisoli et al. 2014) and we had functional virus already produced, we overexpressed E4bp4 in human fibroblasts and cultured them in NK cell differentiation conditions with GBGM. E4bp4 was upregulated on RNA level throughout culture of 19 days. Upregulation of the transcription factors Tox and Id2, which are regulated by E4bp4 during NK cell development (Male, Nisoli et al. 2014), could not be observed. Furthermore, CD56 and IL-15RB were not upregulated during culture. These data suggest that the overexpression of E4bp4 alone in fibroblasts is not sufficient to activate the NK cell specific transcriptional network.

Although our hypotheses could not be confirmed, we have shown that TFs can be efficiently overexpressed in hiPSCs by lentiviral transductions and expression can be maintained over the long differentiation period of 51 days. This would provide a platform for further studies including the overexpression of an array of TFs (e.g. Id2, Tox, Eomes, etc).

We further hypothesized that common lymphoid progenitors (CLPs) would be driven towards NK cell progenitors more efficiently by E4bp4 overexpression. We did not investigate the percentage of CLPs in the MACS enriched CD34 population, but numbers are probably relatively low.

## Chapter 6 General Discussion and Outlook

Harnessing the body's own immune system to fight virally infected or cancerous cells and even autoimmune disease has enormous clinical potential and has already improved the short comes of currently available therapies. The fast moving field of immunotherapy encompasses a variety of approaches including therapeutic antibodies, antigen-specific redirection of cytotoxic lymphocytes via chimeric antigen receptor (CAR) or T cell receptor (TCR) and the adoptive cell transfer of *ex vivo* expanded lymphocytes. NK cell-based immunotherapies have shown great promise in the treatment of various malignancies and different applications are under investigation. The important role of NK cells in the anti-tumour effect of therapeutic antibodies has been well studied, revealing that efficacy occurs via ADCC (Muntasell, Ochoa et al. 2017). Other approaches of NK cell-based therapy include *in vivo* expansion with cytokines, autologous or allogeneic NK cell transfer, the use of NK cell lines and genetically modified NK cells expressing chimeric antigen receptors to improve target cell recognition and cytotoxicity. The need of high numbers of pure and functional NK cells for immunotherapy is critical.

The *in vitro* derivation of large numbers of functional lymphocytes from hPSC sources has huge potential for immunotherapy. In contrast to umbilical cord blood HSCs, hiPSCs can be theoretically expanded unlimited whilst remaining their pluripotent characteristics. This further provides a platform for genetically modifying cells before differentiating them into the lineage of interest whereas genetic modifications on UCB-derived CD34<sup>+</sup> cells are less efficient. The combination of iPSC technology with CRISPR/Cas9-mediated gene editing of autologous cells has enormous potential for treatment of haematopoietic diseases (Hendriks, Warren et al. 2016).



In addition, NK cell therapy with UCB-derived NK cells is dependent on cord blood donations with the desired tissue type. NK cells generated from hPSCs could overcome this limitation by establishing a cell database covering all MHC classes and providing an off-the-shelf cell therapy.

*In vitro* differentiation of haematopoietic precursors from hPSCs has been extensively explored in the literature. Although the differentiation of various mature haematopoietic cells has been proven, the generation of HSCs, which are able to engraft and reconstitute the entire haematopoietic system, remains challenging. hiPSCs could provide an alternative cell source for haematopoietic stem cell transplantation which is used in a variety of malignant and non-malignant haematopoietic disorders. However, these treatments are limited to the amount and quality of HSCs and their differentiation potential. The HLA immuno-compatibility of donor and recipient remains problematic, limiting the access of HSCs for successful therapy. Furthermore, the maturation status of hPSC-derived cells is still a concern. Differentiated cells for transfer could retain oncogenic potential and the use of these cells remains a safety issue. Non-nucleated hPSC-derived cells like erythrocytes and platelets would avoid the risk of tumourigenicity and are therefore extremely suitable for hiPSC-derived cell therapies.

We adopted OP9 co-culture for HSC differentiation from hiPSCs which worked most robust and reproducible in our hands. CD34 is not a unique marker for HSCs, but also for mesenchymal and endothelial cells. HSCs are often characterized by the lack of lineage specific marker and co-expression of CD117, CD133 and CD90. These markers are also expressed in hPSCs which makes it difficult to distinguish hPSCs and HSCs. The assumption that all CD34-expressing cells have haematopoietic differentiation potential – as seen with UCB-derived CD34<sup>+</sup> cells – is probably misleading. Sorting for CD34<sup>+</sup>CD45<sup>+</sup> or CD34<sup>+</sup>CD43<sup>+</sup> double positive cells

could narrow down the HPC fraction. This could have further improved the fold expansion in NK cell differentiation, because the starting cell number of “proper” HPCs would have been lower. On the other side it might be possible that hiPSC-derived CD34<sup>+</sup> cells are primed towards the myeloid lineage at the time of sorting. To address this, cells could be collected at different time points in OP9 co-culture differentiation and could be sorted for an earlier developmental stage like haemogenic endothelium.

Studies of HSC generation with the EB method describe higher efficiencies of HPCs and mature haematopoietic cells can be generated in the absence of feeder cells, serum and cell sorting (Knorr, Ni et al. 2013). Unfortunately, in our hands we could not successfully derive HSCs and NK cells using EB method. Although the differentiation with OP9 co-culture was most robust in our experience, the disadvantage of this method is that the feeder cells and use of serum are non-GMP-compliant. To potentially take this differentiation method to GMP conditions it is of interest to study the mechanism of OP9 co-culture and how haematopoietic development is induced. To investigate if differentiation is induced by cell-cell interactions, experiments using transwell plates could be explored. These plates allow a co-culture of cells - without direct cell-cell contact through a permeable membrane – to identify if differentiation is still facilitated without direct contact. If cell-cell interactions are necessary, the study of receptors and ligands involved will shed light on the mechanism. If secreted factors only play a role, the medium of the feeder cells could be analysed for growth factors and cytokines etc. These studies will help to mimic the differentiation conditions in a GMP-compliant environment without the need of feeder cells.

In addition to these studies, a better understanding of the niche environment *in vivo* and how to recapitulate it *in vitro* will provide vital information. Bone marrow is a

hypoxic environment and future studies could explore hypoxic conditions for differentiation. A better understanding of factors within the bone marrow niche will provide important information for replicating the same environment *in vitro*.

Furthermore, understanding the intrinsic and extrinsic factors that govern haematopoietic development *in vivo* is necessary to mimic conditions in *in vitro* differentiation. This knowledge will help to improve lineage commitment more efficiently by activating certain pathways like Nodal and Wnt signalling or adding factors like BMP4 to induce early specifications in haematopoiesis (reviewed in (Ackermann, Liebhaber et al. 2015)).

In addition, hiPSC-derived HSCs could be in a quiescent or dormant state - comparable to HSCs residing in the BM *in vivo*. By activating certain pathways these cells can temporarily exit this state and begin to actively divide to provide a wider pool of cells for further differentiation. An example is the activation of IFN signalling with an acute dose of IFN- $\alpha$ , which has been shown to stimulate the self-renewal and proliferation of HSCs *in vivo* (Essers, Offner et al. 2009). Furthermore, Notch and Wnt signalling play a fundamental role in haematopoietic self-renewal and differentiation. Notch signalling is important for inhibition of differentiation and Wnt-mediated signalling for keeping HSCs in their undifferentiated state (Duncan, Rattis et al. 2005). Future lines of investigation could include the identification of important pathways for self-renewal to activate hPSC-derived HSCs and test if cell expansion can be improved by adding factors like IFN- $\alpha$  or Notch ligand Jagged1. The comparison of gene expression profiles of hPSC-derived HSCs, UCB-derived and bone marrow HSCs (long-term and short-term) with RNA sequencing could give insights into differences in transcriptional regulations. A cell proliferation assay (e.g. BrdU or CellTrace) could have been helpful to investigate cell proliferation potential.

Despite the low cell expansion of hPSC-derived CD34<sup>+</sup> cells in NK cell differentiation conditions, NK like cells can be generated. It is of importance to note, that NK cell differentiation experiments in this project have been conducted with one hiPS cell line due to the complexity and time constraints of experiments. Importantly, further studies will have to include a variety of hES and hiPS cell lines to take variations between different cell lines into account. Future experiments need to explore the functional properties of hiPSC-derived NK cells. These experiments could include chromium release assays or degranulation assays to test killing efficacy of different tumour cell lines or primary tumour tissue. Furthermore, cytokine release upon activation would be of great interest. In our experiments, the cell surface marker phenotyping with multi-colour flow cytometry was limited by number of fluorochromes in one panel. Future experiments using RNA sequencing will give more insights into gene expression levels of a variety of NK cell specific genes and perforin and granzyme expression upon activation. Furthermore, a detailed comparison of gene expression in hiPSC-derived NK cells compared to UCB-, PBNK and possibly tissue specific NK cells would have been interesting.

To improve cell expansion, future experiments could explore the culture of hPSC-derived NK cells on IL-21 expressing aAPCs. Knorr et al. reported 1000 fold expansion over 70 days of aAPC culture (Knorr, Ni et al. 2013). Taken together, the differentiation process, including expansion, would take over 110 days. Furthermore, the extremely long culture duration in addition to over-activation of NK cells might lead to exhaustion and decreased functional activity of NK cells.

To overcome limitations of conventional *in vitro* HSC differentiation directed by cytokines and morphogens, direct differentiation with transcription factors has been reported in the literature. Doulatov et al. showed that the overexpression of 5 TFs in myeloid progenitors - derived with iPSC-EB differentiation – leads to lymphoid

generation and myeloid and erythroid engraftment potential in mice (Doulatov, Vo et al. 2013). A further study screened a library of 27 TFs to identify candidates able to induce the haematopoietic program. This study shows that the over-expression of ETV2 and GATA2 can induce myeloid and GATA2 and TAL1 can induce erythromegakaryocytic programs in human ESCs and iPSCs (Elcheva, Brok-Volchanskaya et al. 2014).

Transdifferentiation of one somatic cell type to another, without the requirement of reprogramming to a pluripotent state, is an alluring option with less safety concerns. This powerful technique has been applied to converse a variety of somatic cells to other lineages as reviewed in Figure 5.1 (Pfaff and Cantz 2013). This approach further allows to by-pass the limiting step of HSC differentiation. Pereira et al. transduced mouse embryonic fibroblasts (MEFs), which carried a CD34 reporter mechanism, with a library of 18 TFs. Data suggest that Gata2 and Etv6 as well as Gfib and Fos are sufficient to induce the haematopoietic lineage (Pereira, Chang et al. 2013). Another study overexpressed 6 TFs, including Gata2 and Runx1c, which generated haematopoietic progenitors with short-term engraftment *in vivo* (Batta, Florkowska et al. 2014). Transdifferentiation studies have also been performed with human somatic cells. Sandler et al. transduced non-hemogenic umbilical cord and adult endothelial cells with TFs including Runx1 and Gfi-1. Colony forming potential as well as engraftment could be observed in humanized mice after 4 weeks of *in vitro* culture (Sandler, Lis et al. 2014).

Taken together, these studies show the feasibility of TF-driven differentiation and cell fate conversion. Future studies on TF-driven NK cell differentiation will have to include a library of TFs of interest which can be narrowed down at a later stage. Possibly, a combination of NK cell specific and HSC specific TFs could be used to improve lineage specification towards the haematopoietic program.

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